

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
Re: Appeal to the Board of Patent Appeals and Interferences

Appellants:	Vance, et al.)	Examiner:	Vinod Kumar
)		
Serial Number:	10/623,930)	Group Art Unit:	1638
)		
Filed:	July 21, 2003)	Customer Number:	22827
)		
Confirmation No.:	6465)	Deposit Account:	04-1403
)		
Title:	Compositions and Methods for the Modulation of Gene Expression in Plants)	Attorney Docket No.	USC-164
)		

1. ☐ **NOTICE OF APPEAL:** Pursuant to 37 CFR 41.31, Applicant hereby appeals to the Board of Appeals and interferences from the last decision of the Examiner.
2. ☐ **PRE-APPEAL BRIEF REQUEST FOR REVIEW:** Applicant requests review of the final rejection in the above-identified application. No amendments are being filed with this request. This request is being filed with a Notice of Appeal. The review is requested for the reason(s) stated on the attached sheet(s) [No more than five (5) pages may be provided.]
3. ☒ **BRIEF** on appeal in this application pursuant to 37 CFR 41.37 is transmitted herewith (1 copy).
4. ☐ An **ORAL HEARING** is respectfully requested under 37 CFR 41.47 (due within two months after Examiner's Answer).
5. ☐ Reply Brief under 37 CFR 41.41(b) is transmitted herewith (1 copy).
6. ☐ "Small entity" verified statement filed: [] herewith [] previously.

7. FEE CALCULATION:

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SUBTOTAL: \$ 670.00

Less any previous extension fee paid since above original due date. - \$ 0.00

Less any previous fee paid for prior Notice of Appeal since Board did

not render a decision on the merits. MPEP § 1204.01 - \$ 0.00

Less any previous fee paid for submitting Brief on prior Appeal since
Board did not render a decision on the merits. MPEP § 1204.01 - \$ 0.00

SUBTOTAL: \$ 670.00

If "small entity" verified statement filed ☐ previously,
☐ herewith, enter one-half (½) of subtotal and subtract - \$ 335.00

TOTAL FEE ENCLOSED: \$ 335.00

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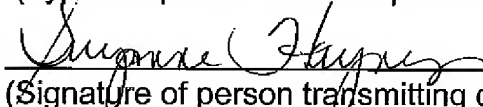
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Date: January 5, 2010

I hereby certify that this correspondence and all attachments and any fee(s) are being electronically transmitted via the internet to the U.S. Patent and Trademark Office using the Electronic Patent Filing System on January 5, 2010.

Suzanne Haynes

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Modulation Of Gene Expression In)	
Plants)	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

BRIEF ON APPEAL

Appellants submit the following Brief on Appeal in accordance with 37 C.F.R. § 41.37:

1. REAL PARTY IN INTEREST

The real party in interest in this matter is the assignee of record, The University of South Carolina.

2. RELATED APPEALS AND INTERFERENCES

There are no other appeals or interferences known to the Appellants or the Appellants' legal representative which will directly affect or be directly affected by or have a bearing on the Board's decision in the pending appeal.

3. STATUS OF CLAIMS

Currently, claims 1-5, 20, 23, and 26 remaining pending including independent claims 1, 20, and 23. Claims 6-19, 21-22, and 24-25 have been previously cancelled from the application. Claims 1-5 stand withdrawn as directed to non-elected inventions. All pending claims are attached hereto in the Claims Appendix.

In the Office Action of June 2, 2009, claims 20, 23, and 26 were rejected.

The rejection of claims 20, 23, and 26 is hereby appealed.

4. STATUS OF AMENDMENTS

To the Appellants' knowledge, all amendments have been entered into the record.

5. SUMMARY OF CLAIMED SUBJECT MATTER

The captioned application claims filing benefit under 35 U.S.C. §120 of U.S. Provisional Patent Application No. 60/397,487 having a filing date of July 19, 2002, a copy of which is provided at Evidence Appendix A. The references to the page and line number in this Summary of Claimed Subject Matter include those for both the regular utility application as filed and the earlier filed provisional application, respectively.

Independent claim 20 is directed to a plant stably transformed (p. 27, ll. 9-21; Provisional p. 31, ll. 19-31) with an miRNA precursor construct (p. 3, ll. 8-24; Provisional p. 3, ll. 1-15). The miRNA precursor construct comprises a promoter functional in a plant cell, and the promoter is operably linked to a nucleotide sequence encoding an isolated plant miRNA precursor (p. 9, ll. 7-11 and p. 22, ll. 12-21; Provisional p. 13, ll. 15-19 and p. 26, ll. 6-14). Additionally, the isolated plant miRNA precursor has been modified by

(a) replacing an endogenous miRNA sequence of the isolated plant miRNA precursor with an exogenous miRNA sequence (p. 9, ll. 18-26; Provisional p. 13, l. 26 – p. 14, l. 3) that maintains the length of the endogenous miRNA sequence (p. 32, ll. 6-23; Provisional p. 36, l. 21 – p. 37, l. 6); and

(b) modifying nucleotides opposite the exogenous miRNA sequence in the isolated plant miRNA precursor to maintain double strandedness and mismatches of the isolated plant miRNA precursor (Examples 3 and 4 (p. 31, l. 17 – p. 35, l. 2); Provisional Examples 3 and 4 (p. 36, l. 1 – p. 39, l. 16)).

Moreover, the exogenous miRNA sequence is complementary to a target mRNA sequence within the plant (p. 31, ll. 22-25; Provisional p. 36, ll. 6-9) and following processing from said plant miRNA precursor, the exogenous miRNA sequence hybridizes with the target mRNA sequence, whereby the expression of the target sequence is reduced (p. 35, ll. 7-14; Provisional p. 39, ll. 21-27).

Independent claim 23 is directed to a plant cell stably transformed (p. 27, ll. 9-21; Provisional p. 31, ll. 19-31) with an miRNA precursor construct (p. 3, ll. 8-24; Provisional p. 3, ll. 1-15). The miRNA precursor construct comprises a promoter functional in a plant cell, and the promoter is operably linked to a nucleotide sequence encoding an isolated plant miRNA precursor (p. 9, ll. 7-11 and p. 22, ll. 12-21; Provisional p. 13, ll. 15-19 and p. 26, ll. 6-14). Additionally, the isolated plant miRNA precursor has been modified by

(a) replacing an endogenous miRNA sequence of the isolated plant miRNA precursor with an exogenous miRNA sequence (p. 9, ll. 18-26; Provisional p. 13,

I. 26 – p. 14, l. 3) that maintains the length of the endogenous miRNA sequence (p. 32, ll. 6-23; Provisional p. 36, l. 21 – p. 37, l. 6); and

(b) modifying nucleotides opposite the exogenous miRNA sequence in the isolated plant miRNA precursor to maintain double strandedness and mismatches of the isolated plant miRNA precursor (Examples 3 and 4 (p. 31, l. 17 – p. 35, l. 2); Provisional Examples 3 and 4 (p. 36, l. 1 – p. 39, l. 16)).

Moreover, the exogenous miRNA sequence is complementary to a target mRNA sequence within the plant (p. 31, ll. 22-25; Provisional p. 36, ll. 6-9) and following processing from said plant miRNA precursor the exogenous miRNA sequence hybridizes with the target mRNA sequence, whereby the expression of the target sequence is reduced (p. 35, ll. 7-14; Provisional p. 39, ll. 21-27).

6. GROUND OF REJECTION TO BE REVIEWED ON APPEAL

Claims 20, 23, and 26 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Cullen, et al. (U.S. Published Patent Application No. 2004/0053411, claiming filing benefit of U.S. Provisional Patent Application 60/337,224 having a filing date of May 3, 2002) in view of Llave, et al. (*The Plant Cell*, 14:1605-1619, 2002) and Reinhart, et al. (*Genes & Development*, 16:1616-1626, 2002).

7. ARGUMENT

The prior filed provisional application to which the pending application claims filing benefit fully supports the rejected claims under the first paragraph of 35 U.S.C. §112, as required. Accordingly, the rejected claims have the same effect as though filed in a regular utility application on the date of the prior provisional application, i.e., July 19, 2002. The regular utility application of Cullen, et al., having a filing date of May

5, 2003, is therefore not proper prior art under 35 U.S.C. §102. As such, the arguments presented below refer only to the provisional application to which the Cullen, et al. regular utility application claims filing benefit, which has a filing date of May 3, 2002,¹ unless specifically indicated otherwise.

1. Claims 20, 23, and 26 patentably define over Cullen, et al., Llave, et al. and Reinhart, et al.

Cullen, et al. relates to artificial miRNAs and to a method of using the artificial miRNAs to specifically inhibit the expression of selected genes in the human and non-human animal cells and in plant cells (p. 2, l. 27- p. 3, l. 1). The miRNA of the construct of Cullen, et al. is excised from a designed precursor that includes a stem-loop structure that is about 40 to 100 nucleotides in length. The stem portion of the construct can be about 19-45 nucleotides and the loop portion can be about 4 but not more than 25 nucleotides in length. It is preferred that the stem comprise a perfectly complementary duplex, but a few (e.g., 1, 2, or 3) bulges can be present that are advantageously about 3 nucleotides or less in size. (P. 6, l. 22 – P. 7, l. 11.)

Reinhart, et al. and Llave, et al. describe miRNAs that had been discovered and categorized prior to the filing of the captioned application. As described in the declaration of Vicki Bowman Vance, Ph.D., previously presented to the Examiner and provided at Evidence Appendix B, miRNAs and the stem-loop precursors from which they are derived were known in the art at the time of the filing of the captioned application (Vance declaration, section 3). Reinhart, et al. and Llave, et al. are examples of the primary literature describing plant miRNA precursors. Thus, existing plant miRNAs and their precursors, as well as the technology used to isolate the

¹ By presentation of these arguments, Appellants in no way admit that the Cullen, et al. provisional application is proper prior art to the captioned application.

miRNAs and precursors were known in the art at the time of filing of the captioned application.

A. Cullen, et al., Llave, et al., and Reinhart, et al., taken either alone or in any proper combination fail to disclose or suggest elements of independent claims 20 and 23.

To establish a *prima facie* case of obviousness, in addition to other requirements, the prior art reference (or references when combined) must teach or suggest all the claim limitations.²

Cullen, et al. includes one example in which an endogenous miRNA precursor is designed to include an exogenous sequence. Beginning at p. 14, l. 26 of Cullen, et al., this example is presented in which a designed miRNA is produced *in vivo*. According to Cullen, et al., the stem sequence of a mir-30 precursor (an miRNA isolated from the human cell line HeLa) was substituted with a sequence based upon the drosophila *nxt* gene. The specific *nxt* sequence was nucleotides 121-143 from the translation initiation codon of drosophila Gene CG10174. The new miRNA precursor was expressed from a human cell line as part of a longer mRNA transcript.

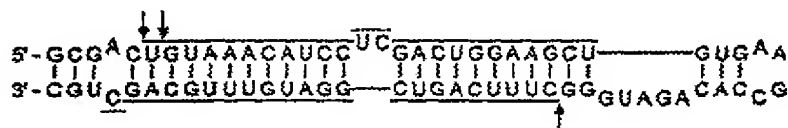
Reinhart, et al. and Llave, et al. describe multiple plant miRNA precursors, but neither reference discloses or suggests the formation of a transformed plant or plant cell that includes an exogenous sequence of any kind.

² *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991).

1. **Cullen, et al., Llave, et al., and Reinhart, et al.**, taken either alone or in any proper combination fail to disclose or suggest a plant or plant cell stably transformed with an miRNA precursor construct, wherein the isolated plant miRNA precursor construct has been modified by replacing an endogenous miRNA sequence of the isolated plant miRNA precursor with an exogenous miRNA sequence that maintains the length of the endogenous miRNA sequence, as is found in independent claims 20 and 23.

The mir-30 precursor utilized by Cullen, et al. is illustrated in Fig. 1A of the provisional patent application, replicated below:

A mir-30 precursor:



As can be seen, the entire precursor includes 71 nucleotides. The precursor includes mature mir-30 on the 3' arm and anti-mir-30 on the 5' arm. These sections of the precursor are indicated by underlining on the above figure. Arrows point to the 5' ends of the mature miRNA and miRNA*. (P. 3, ll. 12-21). As can be seen, the miRNA sequence is 22 nucleotides in length.

The mir-30-nxt precursor formed by Cullen, et al. that includes the exogenous drosophila sequence in place of the endogenous mir-30 miRNA sequence is illustrated in Figure 3A of the provisional patent application and replicated below:

A mir-30-nxt precursor:



The mature mir-30-nxt is underlined on the 3' end and the anti-mir-30-nxt is underlined on the 5' end (p. 4, ll. 14-15; p. 15, ll. 7-12).

As can be seen, the designed precursor includes 67 nucleotides, not the 71 nucleotides of the original mir-30 precursor (see also, SEQ ID NOs: 5 and 8 of the Cullen, et al. regular utility application). Additionally, the miRNA sequence of the mir-30-nxt precursor is 21 nucleotides in length, which differs from the 22 nucleotides of the endogenous sequence.

Cullen, et al. does not maintain the length of the endogenous miRNA sequence, as is required in independent claims 20 and 23 of the captioned application. Moreover, Cullen, et al. does not maintain even the length of the endogenous miRNA precursor. Nowhere in Cullen, et al. is any suggestion made that the designed precursor should maintain the length of the endogenous miRNA sequence, as is found in the pending independent claims 20 and 23. In fact, in the Cullen, et al. regular utility application, it is specifically stated that while the presence of a loop may be desirable, variations of the structure of the precursor can be tolerated (p. 3, ¶ [0023], Cullen, et al. regular utility application).

Neither Llave, et al. nor Reinhart, et al. disclose an artificial construct including an exogenous miRNA sequence. Thus, even if one were to combine the teachings of the references, as was suggested in the Office Action, the combined references would teach the formation of a designed miRNA precursor according to the description provided in Cullen, et al., in which both the length and secondary structure of the endogenous miRNA sequence has been altered. There is no suggestion in any of the references to maintain the length and secondary structure of an endogenous miRNA

sequence following replacement with the exogenous miRNA sequence in an artificial miRNA construct, as is found in independent claims 20 and 23.

2. **Cullen, et al., Llave, et al., and Reinhart, et al., taken either alone or in any proper combination fail to disclose or suggest a plant or plant cell stably transformed with an miRNA precursor construct, wherein the isolated plant miRNA precursor construct has been modified by modifying nucleotides opposite the exogenous miRNA sequence in the isolated plant miRNA precursor to maintain double strandedness and mismatches of the isolated plant miRNA precursor, as is found in independent claims 20 and 23.**

As may be seen in the figures provided above, the Cullen, et al. mir-30-nxt precursor does not retain the mismatches of the isolated miRNA precursor that was utilized in the formation process. Specifically, the designed precursor fails to include a 2 nucleotide bulge in the miRNA sequence portion of the construct stem that is present in the isolated endogenous precursor. Moreover, according to Cullen, et al. it is preferred that the stem comprise a perfectly complementary duplex (p. 7, ll. 3-4). Cullen, et al., as well as Llave, et al. and Reinhart, et al. simply fail to disclose or suggest an miRNA construct in which modification of the nucleotides opposite the exogenous miRNA sequence has been carried out to maintain mismatches of the isolated precursor, as is found in independent claims 20 and 23.

As stated in the Vance declaration at section 5, "to ensure that the newly designed miRNA remains within the proper context of the natural miRNA precursor and is therefore properly processed, the sequences opposite (or complementary) to the artificial miRNA in the stem are also modified to maintain the secondary structure of the stem." Cullen, et al. fails to disclose this concept in forming an miRNA precursor, and

Llave, et al. and Reinhart, et al. fail to disclose the formation of any artificial precursor materials at all.

For at least this reason, Appellants respectfully maintain that claims 20, 23, and 26 patentably define over the cited references.

B. One of ordinary skill in the art would not have combined the teachings of Cullen, et al., Llave, et al., and Reinhart, et al. as attempted by the Office Action.

In conducting an analysis under § 103(a), there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness.³ Accordingly, even if all elements of a claim are disclosed in various prior art references, the claimed invention taken as a whole cannot be said to be obvious without some reason given in the prior art why one of ordinary skill would have been prompted to modify the teachings of the references to arrive at the claimed invention.⁴ The key to supporting any rejection under 35 U.S.C. § 103 is the clear articulation of the reason(s) why the claimed invention would have been obvious.⁵

1. Cullen, et al. teaches away from the invention

The Federal Circuit has several times expressly addressed the issue of how to evaluate an alleged case of prima facie obviousness to determine whether it has been properly made. For instance, “a prima facie case of obviousness can be rebutted if the applicant can show that the art in any material respect taught away from the claimed invention.”⁶

³ *KSR Int'l Co. v. Teleflex Inc.*, 127 S. Ct. 1727, 82 USPQ2d 1385, 1396 (2007); *In re Kahn*, 441 F.3d 977, 988, 78 USPQ2d 1329, 1336 (Fed. Cir. 2006).

⁴ See e.g., *In re Regel*, 188 U.S.P.Q. 132 (C.C.P.A. 1975).

⁵ *KSR Int'l Co. v. Teleflex Inc.*, 127 S. Ct. 1727, 82 USPQ2d 1385, 1396 (2007).

⁶ *In re Haruna*, 249 F.3d 1327, 1335 (Fed. Cir. 2001), citing *In re Geisler*, 116 F.3d 1465, 1469 (Fed. Cir. 1997).

A reference may be said to teach away when a person of ordinary skill, upon reading the reference, would be discouraged from following the path set out in the reference, or would be led in a direction divergent from the path that was taken by the applicant.⁷ Furthermore, a "prior art reference must be considered in its entirety, i.e., as a whole, including portions that would lead away from the claimed invention."⁸

In addition to the references failing to disclose or suggest limitations of the independent claims, as discussed above, Appellants further submit that Cullen, et al. teaches away from the inventions of claims 20, 23, and 26.

The miRNA precursor described in Cullen et al. that includes an exogenous miRNA sequences within an miRNA precursor is shown in Figure 3A, replicated above. As discussed previously, this artificial miRNA clearly does not show an miRNA construct wherein the endogenous miRNA sequence within the stem is replaced with an exogenous miRNA sequence while maintaining the length of the endogenous miRNA sequence and wherein the double strandedness and mismatches of the endogenous miRNA precursor is maintained as found in the pending claims. Figure 3A of Cullen et al. shows a designed miRNA that is missing the bulge of the endogenous miRNA and having an exogenous miRNA sequence length that is different from the endogenous miRNA sequence.

Moreover, Cullen et al. specifically states that "it is preferred that the stem comprise a perfectly complementary duplex." (p. 7, l. 3). Thus, the demonstrated example of forming an miRNA precursor including an exogenous miRNA sequence clearly follows the teaching of the description portion of Cullen, et al. in presenting a

⁷ *In re Gurley*, 27 F.3d 551, 553 (Fed. Cir. 1994).

⁸ M.P.E.P. 8th Ed., Rev. 2, §2141.02, citing *W.L. Gore & Associates v Garlock, Inc.*, 721 F.2d 1540 (Fed. Cir. 1983).

stem that comprises a perfectly complementary duplex through the miRNA sequence, and disregards the bulge of the endogenous miRNA precursor. Based upon the teachings of the reference, it is obvious that the Cullen provisional teaches that maintenance of the length of the miRNA sequence and maintenance of the secondary structure of the miRNA precursor are not necessary.

One of skill in the art, upon reading the Cullen, et al. provisional, would understand that when forming an artificial miRNA construct, the maintenance of the length of the exogenous miRNA sequence and the maintenance of the secondary structure is not necessary. One of skill in the art, when forming an artificial miRNA construct, would follow the teachings of Cullen, et al. and therefore would not maintain the length of the endogenous miRNA sequence in the exogenous sequence and would not maintain the mismatches of the isolated miRNA precursor. Thus, one of skill in the art would be led by Cullen, et al. in a direction divergent from the path that was taken by the Appellants. Clearly, Cullen, et al. teaches away from an miRNA precursor wherein the endogenous miRNA sequence is replaced with an exogenous miRNA sequence that maintains the length of the endogenous miRNA sequence and wherein the mismatches of the isolated miRNA precursor is maintained as is found in independent claims 20 and 23.

It is unclear why the Office Action fails to note that Cullen et al. does not take into consideration and, in fact, teaches away from, maintaining the length and mismatches of the miRNA precursors. Appellants find this particularly surprising since throughout the prosecution of the present application, the Examiner has repeatedly raised the issue of secondary structure in the designing of artificial miRNAs. For example, in the Office

Action mailed October 31, 2006, it is stated that "[d]esigning an miRNA precursor comprising a sequence which is complementary to the target sequence of interest would require prediction analysis of said precursors to form an irregular hairpin structure containing various mismatches, internal loops and bulges so that the predicted precursor molecule forms most favorable structure in solution. This involves extensive analysis of secondary structures of the predicted miRNA precursors and selecting the structure with the lowest free energy so that predicted non-natural miRNA precursor is effectively processed...." Cullen et al. mentions the possibility of including bulges but never within the context of the endogenous miRNA precursor secondary structure and as discussed above, in examples shown in Cullen et al. the artificial miRNA precursors violate one or more aspects of the secondary structure of the native miRNA precursor (see, e.g., Figures 1A, and 3A of Cullen et al.).

Accordingly, Appellants respectfully maintain that Cullen, et al. teaches away from the inventions of claims 20, 23, and 26 and as such, the claims patentably define over the cited references.

2. Cullen, et al., Llave, et al., and Reinhart, et al., taken alone or in any proper combination, fail to enable the inventions of claims 20, 23, and 26.

A conclusion of obviousness requires that the reference(s) relied upon be enabling in that it put the public in possession of the claimed invention.⁹ The court has stated:

Thus, upon careful reconsideration it is our view that if the prior art of record fails to disclose or render obvious a method for making a claimed compound, at the time the invention was made, it may not be legally concluded that the compound itself is in the possession of the public. [footnote omitted.] In this context, we say that the absence of a known or obvious process for making the claimed

⁹ *In re Hoeksema*, 399 F.2d 269, 274, 158 USPQ 596, 601 (CCPA 1968)

compounds overcomes a presumption that the compounds are obvious, based on close relationships between their structures and those of prior art compounds.¹⁰

In the present case, Appellants respectfully submit that the cited art fails to enable the formation of a stably transformed plant or plant cell as is found in independent claims 20 and 23. The Cullen, et al. provisional fails to disclose any plant miRNAs or miRNA precursors and only briefly mentions plants at all. For instance, Cullen, et al. mentions that the invention provides RNA molecules (miRNAs) functionally equivalent to siRNAs that can be transcribed endogenously in animal and plant cells (p. 2, ll. 19-21). Additionally, Cullen, et al. states that the invention relates to artificial miRNAs and to a method of using same to specifically inhibit the expression of selected genes in human and non-human animal cells and in plant cells (p. 2, l. 27 – p. 3, l. 1; p. 5, ll. 23-25; p. 8, ll. 9-11). Cullen, et al. also states that similar approaches can be used in plants to render plants immune to viruses (p. 9, ll. 7-8). However, no examples of artificial nucleic acid constructs including plant miRNA precursors are described in Cullen, et al., and only animal miRNA precursors are discussed in any detail.

As the Office Action mailed October 31, 2006 acknowledges, "differences exist in the structure and production of miRNA precursors from diverse sources, such as, animals, plants, metazoan etc. The differences include cellular compartmentalization, timing of precursor processing or types of cofactors involved in the processing of miRNA." Thus, one of ordinary skill in the art would understand that constructs designed of and for animal miRNA precursors would be quite different from those utilizing plant-based material. Accordingly, one of skill in the art would recognize that given the disclosure of Cullen, et al. in which all detailed information is in regard to

¹⁰ *Ibid.*

animal-based materials, the Cullen, et al. provisional does not teach artificial plant miRNA precursors.

This is borne out by the detailed teachings that are provided in the Cullen, et al. provisional. For instance, in addition to forming an animal-based construct that does not maintain the length or secondary structure of the endogenous precursor, and providing no specific teachings to the formation of transformed plants, Cullen, et al. teaches that the stem-loop structures of the designed miRNA precursors can be about 40 to about 100 nucleotides long (p. 6, l. 8 – p. 7, l. 1). However, virtually all plant miRNA precursors are greater than 100 nucleotides in length (see, e.g., Table 1 of Reinhart, et al.). Thus, not only does Cullen, et al. fail to teach miRNA constructs for utilization in forming transformed plants and plant cells, the animal-based teachings of Cullen, et al. actually contradict the teachings of the secondary references, which describe natural miRNA precursors. Accordingly, even if combined as suggested, Appellants maintain that the combined references fail to enable the transformed plants and plant cells of claims 20, 23, and 26.

C. Only improper hindsight gained from exposure to Appellants' disclosure would lead the person of ordinary skill from Cullen, et al., Llave, et al. and Reinhart, et al. to the limitations of claims 20, 23, and 26.

Plainly, the Examiner's only incentive or motivation for modifying Cullen, et al. using the teachings of Llave, et al. and Reinhart, et al. in the manner suggested in the Office Action results from using Appellants' disclosure as a blueprint to reconstruct the claimed invention out of isolated teachings in the prior art, which is improper under 35 U.S.C. § 103. The U.S. Supreme Court has reaffirmed that "[a] factfinder should be aware, of course, of the distortion caused by hindsight bias and must be cautious of

argument reliant upon *ex post* reasoning.”¹¹ Nevertheless, in KSR the Supreme Court also qualified the issue of hindsight by stating that “[r]igid preventative rules that deny factfinders recourse to common sense, however, are neither necessary under our case law nor consistent with it.”¹²

the Final Office Action asserts that “it would have been obvious and within the scope of an ordinary skill in the art to maintain the secondary structure of the modified plant miRNA precursor because it was unknown what the effect of removing the secondary structure would be, it would have been obvious not to alter it.” Page 7, Final Office Action of September 4, 2008. Besides ignoring the fact that nowhere in the text of Cullen et al., Llave, et al., or Reinhart, et al. is it taught or suggested that maintenance of the mismatches is of concern in forming an artificial miRNA construct, the above statement of the Office Action dismisses the fact that Cullen et al. does just the opposite in altering both the length of the miRNA sequence and in not maintaining the secondary structure of the construct. Moreover, Cullen, et al. explicitly states in the regular utility application that “variations in the structure can be tolerated.” (p. 3, ¶[0023]).

The only indication found to suggest that an miRNA precursor construct should be modified by

(a) replacing an endogenous miRNA sequence of the isolated plant miRNA precursor with an exogenous miRNA sequence that maintains the length of the endogenous miRNA sequence; and

¹¹ *KSR Int’l Co. v. Teleflex Inc.*, 127 S. Ct. 1727, 82 USPQ2d at 1397. See also, *Graham v. John Deere Co.*, 383 U.S. at 36, 148 USPQ at 474.

¹² *KSR Int’l Co. v. Teleflex Inc.*, 127 S. Ct. 1727, 82 USPQ2d at 1397.

(b) modifying nucleotides opposite the exogenous miRNA sequence in the isolated plant miRNA precursor to maintain double strandedness and mismatches of the isolated plant miRNA precursor

is in the captioned application. Only with Appellants' specification could the method of the independent claims be attained, and any attempt to arrive at the method of claims 20, 23, or 26 through study of the cited references is only reachable from improper hindsight analysis after viewing Appellants' specification.

In conclusion, Appellants request favorable action and allowance of the presently pending claims.

Respectfully requested,

DORITY & MANNING, P.A.

Date: 1/5/10


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8. CLAIMS APPENDIX

1. (Withdrawn) A method for producing the plant of claim 20 comprising transforming a plant cell with an miRNA precursor construct, said construct comprising a promoter functional in a plant cell, wherein the promoter is operably linked to a nucleotide sequence encoding an isolated plant miRNA precursor, wherein the isolated plant miRNA precursor has been modified by

(a) replacing an endogenous miRNA sequence of the isolated plant miRNA precursor with an exogenous miRNA sequence that maintains the length of the endogenous miRNA sequence; and

(b) modifying nucleotides opposite the exogenous miRNA sequence in the isolated plant miRNA precursor to maintain double strandedness and mismatches of the isolated plant miRNA precursor,

and further wherein the exogenous miRNA sequence is complementary to a target mRNA sequence within said plant and, following processing from said precursor, hybridizes with the target mRNA sequence, whereby the expression of the target sequence is reduced.

2. (Withdrawn) The method of claim 1, wherein said target sequence is an endogenous plant sequence.

3. (Withdrawn) The method of claim 1, wherein said target sequence is an exogenous sequence.

4. (Withdrawn) The method of claim 1, wherein said target sequence is selected from the group consisting of genes involved in the synthesis and/or degradation of proteins, peptides, fatty acids, lipids, waxes, oils, starches, sugars,

carbohydrates, flavors, odors, toxins, carotenoids, hormones, polymers, flavinoids, storage proteins, phenolic acids, alkaloids, lignins, tannins, celluloses, glycoproteins, and glycolipids.

5. (Withdrawn) The method of claim 1, wherein said promoter is selected from the group consisting of a constitutive promoter, tissue-preferred promoter, and an inducible promoter.

6-19. (Canceled).

20. (Rejected) A plant stably transformed with an miRNA precursor construct, said miRNA precursor construct comprising a promoter functional in a plant cell, wherein the promoter is operably linked to a nucleotide sequence encoding an isolated plant miRNA precursor, wherein the isolated plant miRNA precursor has been modified by

(a) replacing an endogenous miRNA sequence of the isolated plant miRNA precursor with an exogenous miRNA sequence that maintains the length of the endogenous miRNA sequence; and

(b) modifying nucleotides opposite the exogenous miRNA sequence in the isolated plant miRNA precursor to maintain double strandedness and mismatches of the isolated plant miRNA precursor,

and further wherein the exogenous miRNA sequence is complementary to a target mRNA sequence within said plant and, following processing from said plant miRNA precursor, hybridizes with the target mRNA sequence, whereby the expression of the target sequence is reduced.

21-22. (Canceled).

23. (Rejected) A plant cell stably transformed with an miRNA precursor construct, said miRNA precursor construct comprising a promoter functional in a plant cell, wherein the promoter is operably linked to a nucleotide sequence encoding an isolated plant miRNA precursor, wherein the isolated plant miRNA precursor has been modified by

(a) replacing an endogenous miRNA sequence of the isolated plant miRNA precursor with an exogenous miRNA sequence that maintains the length of the endogenous miRNA sequence; and

(b) modifying nucleotides opposite the exogenous miRNA sequence in the isolated plant miRNA precursor to maintain double strandedness and mismatches of the isolated plant miRNA precursor,

and further wherein the exogenous miRNA sequence is complementary to a target mRNA sequence within said plant and, following processing from said plant miRNA precursor, hybridizes with the target mRNA sequence, whereby the expression of the target sequence is reduced.

24-25. (Canceled).

26. (Rejected) Transformed seed of the plant of claim 20.

9. **EVIDENCE APPENDIX**

See following

Appendix A

07/19/02
1c904 U.S. PTO

Attorney's Docket No. 46795/250579
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07-22 68397487 07/19/02 A/prov.

PATENT

COVER SHEET FOR FILING PROVISIONAL PATENT APPLICATION

Box Provisional Patent Application
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Washington, DC 20231

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60/397487
07/19/02

This is a request for filing a PROVISIONAL PATENT APPLICATION under 37 C.F.R. 1.53(c).

Docket No.	46795/250579
Type a plus sign (+) inside this box →	+

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TITLE OF THE INVENTION (280 characters maximum)

Compositions and Methods for the Modulation of Gene Expression in Plants

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ENCLOSED APPLICATION PARTS (check all that apply)

- ☒ Specification (Number of Pages 48)
- ☒ Drawing(s) (Number of Sheets 4)
- ☐ Claims (Number of Claims 1)
(A complete provisional application does not require claims 37 C.F.R. § 1.51(a)(2))
- ☐ Application Data Sheet. See 37 CFR 1.76
- ☐ CD(s), Number
- ☐ Other (specify)

Attorney Docket No. 46795/250579

Filed: Concurrently Herewith

Page 2

METHOD OF PAYMENT (check one)

- ☐ Applicant claims small entity status
- ☒ Check or money order is enclosed to cover the filing fee.
- ☐ The Commissioner is hereby authorized to charge filing fees and credit Deposit Account No. 16-0605.
- ☒ Please charge Deposit Account No. 16-0605 for any fee deficiency.

PROVISIONAL FILING FEE AMOUNT(s)

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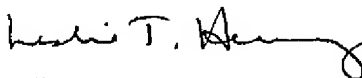
Small Entity \$ 80.00

Filing Fee Amount: \$160.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

- ☐ No.
- ☒ Yes, the name of the U.S. Government agency and the Government contract number are: USDA grant/project no. 200113531909848, account no. 13010-FA04, NIH grant/project no. R-02-0927, account no. 13010-FA09.

Respectfully submitted,



Leslie T. Henry
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Date: July 19, 2002

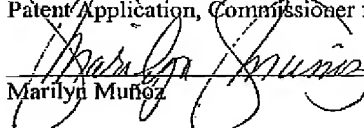
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Marilyn Muñoz

Attorney Docket No. 46795/250579

COMPOSITIONS AND METHODS FOR THE MODULATION
OF GENE EXPRESSION IN PLANTS

FIELD OF THE INVENTION

5 The invention relates to compositions and methods for modulating gene expression in plants.

BACKGROUND OF THE INVENTION

Gene transfer is also being utilized to modify the quality of harvested products to
10 maximize their use as food or industrial raw material. In the same manner, attempts to alter the amino acid composition of storage proteins to increase their nutritional value have been reported. These efforts have been met with mixed results.

Transgenic plants are also suitable for producing peptides and proteins used as pharmaceuticals, such as enkephalins, human serum albumins, or interferons. The
15 production in transgenic plants of vaccines for use against various illnesses is being considered to reduce production costs. Additionally, transgenic plants can be engineered to produce a wide array of metabolites. Transgenic plants have been used to produce a variety of metabolites including biodegradable plastics know as polyhydroxyalkanoates, various oils that are useful for both human consumption and industrial purposes and
20 carbohydrates.

Many factors affect gene expression in plants and other eukaryotic organisms. Recently, small RNAs, 21-26 nucleotides, have emerged as important regulators of eukaryotic gene expression. The known small regulatory RNAs fall into two basic classes. One class of small RNAs is the short interfering RNAs (siRNAs). These play
25 essential roles in RNA silencing, a sequence-specific RNA degradation process that is triggered by double-stranded RNA (dsRNA) (see Vance and Vaucheret, 2001 and

Zamore, 2001 for recent reviews on RNA silencing in plants and animals, respectively). RNA silencing plays a natural role in defense against foreign nucleic acids including virus resistance in plants and control of transposons in a number of organisms. siRNAs are double-stranded with small 3' overhangs and derive from longer dsRNAs that induce
 5 silencing. They serve as guides to direct destruction of target RNAs and have been implicated as primers in the amplification of dsRNA via the activity of a cellular RNA dependent RNA polymerase. In plants, si-like RNAs have also been associated with dsRNA-induced transcriptional gene silencing (TGS), a process in which dsRNA with homology to promoter regions triggers DNA methylation and inhibits transcription. The
 10 TGS-associated small RNAs, unlike true siRNAs, are not involved in RNA degradation.

The second known class of small RNAs is the small temporal RNAs (stRNAs), *lin-4* and *let-7*, that control certain developmental switches in *C. elegans*. The stRNAs are single-stranded, although they derive from larger precursor RNAs that are partially double-stranded. StRNAs are also functionally different from siRNAs: they interact with
 15 the 3'-untranslated end of target mRNA and inhibit translation rather than mediating RNA degradation. Unlike siRNAs, the stRNAs are only partially complementary to their target mRNAs. Remarkably, hundreds of new stRNA-like small RNAs termed microRNAs (miRNAs) have been discovered in worms, flies, humans, and plants and miRNAs are likely to be discovered in other organisms as well. Like stRNAs, the
 20 miRNAs are single-stranded, and their accumulation is developmentally regulated. They derive from partially double-stranded precursor RNAs that are transcribed from genes that do not encode protein. Like stRNAs (and unlike siRNAs involved in RNA silencing), most of the miRNAs lack complete complementarity to any putative target mRNA. Although their functions are, as yet, not known, it is hypothesized that they
 25 regulate gene expression during development, perhaps at the level of development. However, given the vast numbers of these small regulatory RNAs, it is likely that they are functionally more diverse and regulate gene expression at more than one level.

SUMMARY OF THE INVENTION

30 Compositions and methods for modulating nucleotide sequence expression, particularly for modulating gene expression in plants, are provided. The compositions

comprise precursor RNA constructs for the expression of an RNA precursor. The precursor RNA construct comprises a promoter that is expressed in a plant cell driving the expression of a precursor RNA having a microRNA. The RNA precursor is cleaved in a plant cell to form small temporal RNAs or microRNAs (miRNAs). MicroRNAs (miRNAs) are small regulatory RNAs that control gene expression. The miRNA is complementary or partially complementary to a portion of a target gene or nucleotide sequence and function to modulate expression of the target sequence or gene. In this manner, the RNA precursor construct can be designed to modulate expression of any nucleotide sequence of interest, either an endogenous plant gene or alternatively a transgene. The RNA precursor is designed to produce a transcript that is processed via the miRNA pathway to produce an miRNA complementary to a portion of RNA, the target RNA, that corresponds to the target gene. The miRNA modulates the expression of the target gene. While not bound by any mechanism of action, the miRNA may function to alter the production, processing, stability, or translation of the target RNA and thereby alter the expression of the product of the targeted RNA.

The precursor RNA constructs may be used in combination with modulators to enhance the effect on gene expression. Modulators are proteins of the invention alter the level of at least one miRNA in a plant cell, including, but not limited to plant and viral proteins that are known to alter RNA silencing. Expression of a modulator in the presence of the precursor RNA alters the accumulation of miRNAs and thus enhances the regulatory capabilities of miRNAs. In this manner, a plant expressing both the precursor RNA and a modulator can be constructed to modulate expression of a target gene.

A third embodiment of the invention comprises the use of a modulator to control gene expression via the siRNA and the miRNA pathway. The siRNA pathway involves RNA silencing through the accumulation of short interfering RNAs (siRNAs). These siRNAs incorporate into a silencing complex and guide sequence-specific RNA degradation. Any mRNA with sequence relatedness to the siRNA is targeted and destroyed, effectively silencing the gene. In this embodiment, the plant modulators of the invention interfere with the accumulation of siRNAs and prevent RNA silencing, providing for increased expression of the unsilenced sequence. In this manner, modulators have been used to suppress silencing of a gene or sequence of interest.

Modulators have additionally been used in combination with amplicons to increase the expression of a target gene. An amplicon comprises a targeting sequence corresponding to the gene of interest, the target gene, and directs gene silencing of a sequence having homology to the targeting sequence. The amplicon may optionally comprise a promoter and a sequence that corresponds to at least a part of a viral genome. In this embodiment, the modulator can be used to enhance the regulatory effect of an miRNA and at the same time enhance expression of an amplicon silenced gene. In this embodiment, a plant expresses a modulator, a precursor RNA having miRNA corresponding to a first target sequence, and an amplicon comprising a targeting sequence corresponding to a second target sequence. The plant will demonstrate enhanced expression of the second target sequence and modified expression of the first target sequence.

It is recognized that a variety of promoters may be utilized in the constructs of the invention depending on the desired outcome. Tissue-preferred promoters, inducible promoters, developmental promoters, constitutive promoters can be used to direct expression of the first target sequence, the second target sequence or the modulator sequence in the plant.

Embodiments of the invention include:

1. A method for modulating the expression of a first target sequence in a plant cell, said method comprising transforming said plant cell with an RNA precursor construct, said construct comprising a first promoter that drives expression in a plant cell operably linked to a first nucleotide sequence encoding a precursor RNA, said precursor having at least one miRNA sequence incorporated into the precursor RNA sequence, wherein said miRNA sequence is complementary to a portion of said first target sequence.
2. The method of 1, wherein said first target is an endogenous plant sequence.
3. The method of 1, wherein said first target is an exogenous sequence.
4. The method of 1, wherein said first target sequence is selected from the group consisting of genes involved in the synthesis and/or degradation of proteins, peptides, fatty acids, lipids, waxes, oils, starches, sugars, carbohydrates, flavors,

odors, toxins, carotenoids, hormones, polymers, flavinoids, storage proteins, phenolic acids, alkaloids, lignins, tannins, celluloses, glycoproteins, and glycolipids.

- 5 5. The method of 1, wherein said first promoter is selected from the group consisting of a constitutive promoter, tissue-preferred promoter, and an inducible promoter.
6. The method of 1-5, wherein said plant cell further comprises a DNA construct comprising a second promoter that drives expression in a plant cell operably linked to a second nucleotide sequence encoding a modulator.
- 10 7. The method of 6, wherein said modulator is selected from the group consisting of HC-Pro, the 2b protein of cucumber mosaic virus (CMV), HC-Pro of potato virus Y (PVY), and rg-CaM.
8. The method of 7, wherein said second promoter is selected from the group consisting of a constitutive promoter, tissue-preferred promoter, and an inducible promoter.
- 15 9. The method of 6, wherein said plant cell further comprises an amplicon, said amplicon comprising a targeting sequence that corresponds to a second target sequence.
10. The method of 9, wherein said second target sequence is an endogenous plant sequence.
- 20 11. The method of 9, wherein said second target sequence is an exogenous plant sequence.
12. The method of 10, wherein said second target sequence is selected from the group consisting of those involved in agronomic traits, disease resistance, herbicide resistance, and grain characteristics.
- 25 13. The method of 9, wherein said second target sequence is selected from the group consisting of genes responsible for the synthesis of proteins, peptides, fatty acids, lipids, waxes, oils, starches, sugars, carbohydrates, flavors, odors, toxins, carotenoids, hormones, polymers, flavonoids, storage proteins, phenolic acids, alkaloids, lignins, tannins, celluloses, glycoproteins, and glycolipids.
- 30 14. The method of 11, wherein said second target sequence is selected from the group consisting of retinoblastoma protein, p53, angiostatin, leptin, hormones, growth

- factors, cytokines, insulin, growth hormones, alpha-interferon, beta-glucocerebrosidase, serum albumin, hemoglobin, and collagen.
15. The method of 14, wherein said second target sequence encodes a mammalian protein.
 - 5 16. The method of any of 9-15, wherein said amplicon further comprises a DNA sequence corresponding to at least a portion of a viral genome.
 17. The method of 16, wherein said amplicon further comprises a third promoter that drives expression in a plant cell operably linked to the targeting sequence.
 18. The method of 17, wherein said third promoter is selected from the group
 - 10 consisting of a constitutive promoter, tissue-preferred promoter, and an inducible promoter.
 19. The method of 18, wherein said first target sequence or said second target sequence is selected from the group consisting of genes responsible for the synthesis of proteins, peptides, fatty acids, lipids, waxes, oils, starches, sugars,
 - 15 carbohydrates, carotenoids, hormones, and storage proteins.
 20. A plant stably transformed with an RNA precursor construct, said RNA precursor construct comprising a first promoter that drives expression in a plant cell operably linked to a first nucleotide sequence encoding a precursor RNA, said precursor having at least one miRNA sequence incorporated into the precursor
 - 20 RNA sequence, wherein said miRNA sequence is complementary to a portion of a first target sequence.
 21. The plant of 20, further comprising a DNA construct comprising a second promoter that drives expression in a plant cell operably linked to a second nucleotide sequence encoding a modulator.
 - 25 22. The plant of 21, further comprising an amplicon, said amplicon comprising a targeting sequence that corresponds to a second target sequence.
 23. A plant cell stably transformed with an RNA precursor construct, said RNA precursor construct comprising a first promoter that drives expression in a plant cell operably linked to a first nucleotide sequence encoding a precursor RNA, said precursor having at least one miRNA sequence incorporated into the precursor
 - 30

RNA sequence, wherein said miRNA sequence is complementary to a portion of said first target sequence.

24. The plant cell of 23, further comprising a DNA construct comprising a second promoter that drives expression in a plant cell operably linked to a second nucleotide sequence encoding a modulator.

25. The plant cell of 24, further comprising an amplicon, said amplicon comprising a targeting sequence that corresponds to a second target sequence.

26. Transformed seed of the plant of any one of 20-22.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts that HC-Pro suppression of IR- and amplicon-induced RNA silencing prevents the accumulation of siRNAs and results in accumulation of a new size class of small RNAs. Figure 1A is a diagram of the transgene loci in lines 6b5, T4 and 155 as predicted by DNA blot analysis. 35S indicates the position of the cauliflower mosaic virus (CaMV) 35S promoter, and nos indicates the position of the nopaline synthase terminator. Arrows indicate the direction of transcription in line T4 based on the predicted arrangement of the 35S promoters (Hobbs *et al.*, 1990). The amplicon transgene in line 155 encodes a potato virus X (PVX) complementary DNA in which the coat protein gene is replaced by the GUS locus. The PVX viral open reading frames (ORFs) encode RNA-dependent RNA polymerase (RdRp), and three movement proteins, p25 (25), p12 (12), and p8 (8) (Angell and Baulcombe, 1997). Figure 1B depicts the results of an RNA gel blot analysis of small RNAs from silenced transgenic lines and lines in which silencing has been suppressed by HC-Pro. Lanes 1, 2, 4, 6 and 10 show small RNAs from IR- silenced tobacco line T4 (lanes 1 and 2, lane 1 is a longer exposure of lane 2), sense transgene- silenced tobacco line 6b5 (lane 4 and 10) and an amplicon-silenced tobacco line 155 (lane 6). Lanes 3, 5, 7, 9 and 11 show small RNAs from an IR line expressing HC-Pro (T4 X HC-Pro, lane 3), a sense transgene line expressing HC-Pro (6b5 X HC-Pro, lane 5 and 11) and an amplicon line expressing HC-Pro (155 X HC-Pro, lane 7, lane 9 is a shorter exposure of lane 7). The probe was ³²P labeled RNA

corresponding to the sense strand of the 3' 700-nt of the GUS coding sequence and detects antisense strand GUS RNAs. The migration of 21, 23 and 26 nt DNA oligomers is shown in lane 8. Ethidium bromide (EtBr) staining of the predominant RNA species in the fractionated sample is shown as a loading control. 20 ug of low molecular weight RNA were loaded in each lane, except for lanes 7 and 9 (155 X HC-Pro) in which 5 ug were loaded and lane 11 in which 240 ug were loaded. Figure 1C depicts the results of an RNA gel blot analysis of small RNAs from the same samples shown in Figure 1B. The probe was ³²P labeled RNA corresponding to the antisense strand of the 3' 700 nt of the GUS coding sequence and detects sense-strand, GUS RNAs. Ethidium bromide (EtBr) staining of the predominant RNA species in the fractionated sample is shown as a loading control. 20 ug of low molecular weight RNA were loaded, except for lanes 6 and 7 (155 X HC-Pro) in which 7 ug were loaded.

Figure 2 depicts that HC-Pro suppression of both IR and amplicon but not sense transgene-induced RNA silencing results in the accumulation of full-length GUS dsRNA. Figure 2A depicts the results of an RNA gel blot analysis showing the level of GUS RNA prior to and after RNase A digestion in silenced lines T4 (lane 1 and 2), 155 (lanes 3 and 4), 6b5 (lanes 5 and 6) and a GUS-expressing control line T19 (lanes 7 and 8). 25 ug of total RNA was digested for each plant line. Ethidium bromide staining of 25S rRNA is shown as a loading control. Figure 2B depicts the results of an RNA gel blot analysis showing the level of GUS RNA prior to and after RNase A digestion in plant lines T4 X HC-Pro (lanes 1, 2 and 3), 155 X HC-Pro (lanes 4, 5 and 6), 6b5 X HC-Pro (lanes 7, 8 and 9) and the GUS expressing line T19 X HC-Pro (lanes 10, 11 and 12). The position of GUS viral RNA and subgenomic RNAs (sgRNAs) is indicated. 25 ug of total RNA was digested for each plant line. Heat refers to boiling the samples immediately prior to RNase A digestion to denature dsRNA. Ethidium bromide staining of 25S rRNA is shown as a loading control. Figure 2C depicts the results of an RNA gel blot analysis showing the level of GUS mRNA prior to and after RNase A digestion in silenced line 6b5 (lanes 1, 2 and 3) and the unsilenced line 6b5 X HC-Pro (lanes 4, 5 and 6). 100 ug of total RNA was digested for each plant line and 10 ug of total RNA was used for untreated sample. The heat control is described in panel (B). Ethidium bromide staining of 25S rRNA is shown as a loading control.

Figure 3 depicts that HC-Pro expression leads to increased miRNA accumulation in tobacco. Figure 3A depicts the results of an RNA gel blot analysis of 20 ug of small RNAs from leaf tissue of the silenced line 6b5 (lanes 1, 3, 5, 7 and 9), the HC-Pro expressing line 6b5 X HC-Pro (lanes 2, 4, 6 and 8) and the GUS expressing control line T19 (lane 10). The specific probe used to detect each miRNA is noted (miR167, miR164, miR156 and miR171). The migration of 21 and 25 nt DNA oligomers is shown on the left and ethidium bromide (EtBr) staining of the predominant RNA species in the fractionated sample is shown as a loading control. Figures 3B and 3C depict the results of the RNA gel blot analysis of miR167 and miR164 miRNAs, extracted from flower (F), leaf (L) and stem (St) tissue of a control line Xanthi (lanes 1-3) and the HC-Pro-expressing line X-27-8 (lanes 4-6). The migration of 21 and 25 nt DNA oligomers is shown on the left. Ethidium bromide (EtBr) staining of the predominant RNA species in the fractionated sample is shown as a loading control.

Figure 4 is a schematic illustration of proposed RNA silencing and miRNA biogenesis pathways showing steps that could be differentially regulated by HC-Pro.

DETAILED DESCRIPTION OF THE INVENTION

Compositions and methods for modulating target sequences in a plant are provided. The target sequences may be endogenous or exogenous plant sequences. In one aspect of the invention target sequences are modulated via the microRNA (miRNA) pathway or alterations of this pathway. MicroRNAs (miRNAs) are small regulatory RNAs that control gene expression. miRNAs bind to regions of target RNAs and inhibit their translation (thereby interfering with production of the protein encoded by the target mRNA). Thus, miRNAs of the invention will be complementary to the target gene or sequence of interest. The miRNA can be designed to be complementary to any region of the target sequence RNA including the 3' untranslated region, coding region, etc. miRNAs are processed from highly structured RNA precursors that are processed by the action of a ribonuclease III termed DICER. While the mechanism of action of these miRNAs is currently unknown, they function to regulate expression of the target gene. In

this manner, the pathway that produces miRNAs can be manipulated to control regulation of specific target genes.

In this embodiment, a precursor RNA construct is designed to produce a transcript that would be processed via the miRNA pathway to produce an miRNA complementary to a target RNA, an RNA corresponding or transcribed by the target sequence. While not
5 bound by any mechanism of action, the miRNAs alter the production, processing, stability, or translation of the target RNA and thereby alter the expression of the protein product of the targeted RNA. The miRNAs of the invention will be complementary or substantially complementary to a target RNA that corresponds to the target gene of
10 interest. The miRNA will generally be small molecules comprising about 15 to about 30 nucleotides, about 20 to about 28 nucleotides, more specifically about 21-24 nucleotides. Generally the miRNA will be completely complementary to the target RNA, however, mismatches may be tolerated. Generally from 1-about 6 mismatches may occur, more specifically about 2-3 mismatched nucleotides may be included in the miRNA. While the
15 mismatched nucleotides may occur throughout the miRNA sequence, preferably, they are located near the center of the molecule. In this manner, an miRNA sequence can be designed to modulate the expression of any target sequence. The miRNA is expressed as part of a precursor RNA construct. Such precursor RNA molecules are known in the art. See, for example, Reinhart *et al.* (2002) *Genes & Development* 16:1616-1626 and Llave
20 *et al.* (2002) *Plant Cell* 14:1605-1619, herein incorporated by reference. As noted above, once the precursor RNA construct is expressed in the plant cell, it is processed to produce the miRNAs.

The regulation of a gene via miRNA can be used in combination with a modulator. Such modulators include, but are not limited to, viral (or cellular) proteins
25 that regulate miRNA accumulation. The modulators of the invention are capable of altering the levels of at least one miRNA in a plant. For example, HC-Pro, a viral suppressor of RNA silencing, enhances the accumulation of endogenous miRNAs. Thus, the modulators of the invention can be used in combination with the RNA precursor constructs of the invention to enhance the regulatory capabilities of miRNA that
30 correspond to target sequences of interest.

Modulators of the invention may regulate the miRNA pathway only, or like HC-Pro, may participate in both the miRNA and the siRNA pathways. Modulators of the siRNA pathway have been previously shown to suppress RNA silencing and thereby boost expression of genes that have been targeted by the silencing mechanism, such as, example, HC-Pro. RNA silencing involves the accumulation of short interfering RNAs (siRNAs) that incorporate into a silencing complex and guide sequence-specific RNA degradation. Any mRNA with sequence relatedness to the siRNAs is targeted and destroyed, resulting in failure to produce the encoded protein (and thus effectively silencing the gene). Modulators of the invention interfere with the accumulation of siRNAs and thereby prevent RNA silencing, allowing the targeted gene to be expressed.

In the present invention, a modulator is used to alter regulation of the miRNA pathway. The modulator may work to enhance the accumulation of small regulatory RNAs called miRNAs. Though both siRNAs and miRNAs control gene expression, the biogenesis of these two classes of small RNAs is different and the modulators of the invention may affect their accumulation in different ways.

It is further recognized that gene expression in a plant expressing a modulator that acts on both pathways may be manipulated using both the miRNA and the siRNA pathways. That is, the plant expressing a modulator could be further designed to express a precursor RNA construct of interest that modulates the expression of a first target sequence via the miRNA pathway and to express an amplicon (wherein the amplicon comprises at least a promoter that drives expression in a plant cell operably linked to a targeting sequence that corresponds to a second target sequence of interest) that modulates the expression of a second target sequence via the siRNA pathway. The second target sequence would be highly expressed and the expression of the first target sequence would be altered or modified. Expression of the first target sequence controlled via the miRNA pathway could be effectively silenced (their expression reduced) or alternatively increased. The modulators of the invention may act as both an enhancer and a suppressor of gene expression depending on the circumstances.

The methods of the invention involve modulating the expression of one, two, or more target nucleotide sequences in a plant are provided. That is, the expression of a target nucleotide sequence of interest may be increased or decreased. By "increased

expression" is intended that expression of the target nucleotide sequence is increased over expression observed in conventional transgenic lines for heterologous genes and over endogenous levels of expression for homologous genes. Heterologous or exogenous genes comprise genes that do not occur in the plant of interest in its native state.

- 5 Homologous or endogenous genes are those that are natively present in the plant genome. Generally, expression of the target sequence is substantially increased. That is expression is increased at least about 25%-50%, preferably about 50%-100%, more preferably about 100%, 200% and greater.

- 10 By "decreased expression" is intended is intended that expression of the target nucleotide sequence is decreased below expression observed in conventional transgenic lines for heterologous genes and below endogenous levels of expression for homologous genes. Generally, expression of the target nucleotide sequence of interest is substantially decreased. That is expression is decreased at least about 25%-50%, preferably about 50%-100%, more preferably about 100%, 200% and greater.

- 15 The invention relates to the regulation of gene expression via mechanisms that involve small regulatory RNAs, particularly siRNAs and miRNAs. "Gene silencing", which is also known as "RNA silencing", is generally used to refer to suppression of expression of a gene and involves siRNAs. The degree of reduction may be partial or total reduction in production of the encoded gene product. Therefore, the term should not
20 be taken to require complete "silencing" of expression. Methods for gene silencing are known in the art and include co-suppression and antisense suppression. See, for example, PCT/GB 98/00442 and PCT/GB 98/02862, herein incorporated by reference.

- The methods of the invention relates to modulating the expression of one, two, or more target nucleotide sequences or genes in plants. The target nucleotide sequences
25 may be endogenous or exogenous in origin. By "modulate expression of a target gene" is intended that the expression of the target gene is increased or decreased relative to the expression level in a plant that has not been altered by the methods described herein. Expression levels may be assessed by determining the level of a gene product by any method known in the art including, but not limited to determining the levels of the RNA
30 and protein encoded by a particular target gene. For genes that encode proteins, expression levels may determined, for example, by quantifying the amount of the protein

present in plant cells, or in a plant or any portion thereof. Alternatively, if desired target gene encodes a protein that has a known measurable activity, then activity levels may be measured to assess expression levels.

The target nucleotide sequence comprises any nucleotide sequence or gene of interest, including genes, regulatory sequences, etc. Genes of interest include those encoding agronomic traits, insect resistance, disease resistance, herbicide resistance, sterility, grain characteristics, and the like. The genes may be involved in metabolism of oil, starch, carbohydrates, nutrients, etc. Genes or traits of interest include, but are not limited to, environmental- or stress- related traits, disease-related traits, and traits affecting agronomic performance. Target sequences also include genes responsible for the synthesis of proteins, peptides, fatty acids, lipids, waxes, oils, starches, sugars, carbohydrates, flavors, odors, toxins, carotenoids, hormones, polymers, flavonoids, storage proteins, phenolic acids, alkaloids, lignins, tannins, celluloses, glycoproteins, glycolipids, etc.

The compositions comprise precursor RNA constructs for the expression of an RNA precursor. The precursor RNA construct comprises a promoter that is expressed in a plant cell driving the expression of a nucleotide sequence that encodes the precursor RNA having a microRNA. The RNA precursor is cleaved in a plant cell to form miRNAs. The miRNA is complementary to a portion of a target gene or nucleotide sequence and function to modulate expression of the target sequence or gene. The precursor RNA constructs are designed to direct the expression in the plant an RNA precursor that has an miRNA that is complementary to a portion of a target nucleotide sequence. Such precursor RNAs, their respective miRNAs and the genes that encode them are known in the art and have been identified in plants. See, Reinhart *et al.* (2002) *Genes & Development* 16:1616-1626 and Llave *et al.* (2002) *Plant Cell* 14:1605-1619. The nucleotide sequence that encodes the precursor RNA that comprises an miRNA region that is complementary to a portion of the target gene. The regions which flank the miRNA region are selected from the sequences known in the art for miRNA precursors, particularly plant miRNA precursors, more particularly those plant miRNA precursors disclosed by Reinhart *et al.* (2002) *Genes & Development* 16:1616-1626 and Llave *et al.* (2002) *Plant Cell* 14:1605-1619. In general, an RNA precursor is constructed by

obtaining the sequence of known RNA precursor for an miRNA and replacing the miRNA sequences therein with the miRNA sequences directed to the target gene of interest. Methods for constructing precursor miRNAs and miRNAs that can be used to alter the expression of specific target genes are known in the art. See, for example,

5 McManus *et al.* (2002) *RNA* 8:842-850. Alternatively, precursor miRNAs can be isolated by methods known in the art (Reinhart *et al.* (2002) *Genes & Development* 16:1616-1626 and Llave *et al.* (2002) *Plant Cell* 14:1605-1619).

For a target gene of interest, the miRNA of the invention is complementary or partially complementary to a region of the target gene. That is the miRNA comprises a region that is completely complementary to a region of the target gene, or the miRNA

10 comprises a region that is partially complementary to a region of the target gene. By partially complementary, it is intended the corresponding regions of the target gene and the miRNA have one, two, three, or more mismatched bases. It is recognized in the art that miRNAs may not be completely complementary to the region of a target gene.

15 The miRNAs of the invention comprise a region of that is complementary or partially complementary to a region of the target gene. While the invention does not depend on miRNAs of a particular size, the miRNAs known in the art typically comprise between about 15 and 30 nucleotides.

For purposes of the invention, a nucleotide sequence encoding a modulator is

20 utilized in combination with a nucleotide sequence encoding a precursor miRNA. The modulator is capable of modulating the level of at least one miRNA in a plant cell, and may also be capable of suppressing co-suppression in plants, such as, for example, the silencing of an inverted repeat transgene, and amplicon transgene or a sense transgene. See, for example, PCT/US 98/06075, herein incorporated by reference. As discussed

25 above, miRNAs are involved in regulating gene expressions in organisms. In fact, synthetic miRNA can either be transfected into cells or expressed in the cell under the control of an RNA polymerase III promoter and cause the decreased expression of a specific target nucleotide sequence (McManus *et al.* 2002, herein incorporated by reference). While the instant invention does not depend on a particular biological

30 mechanism for miRNAs modulating gene expression, such miRNAs are not believed to

be involved in RNA silencing, which involves a sequence-specific RNA degradation process and siRNAs that is triggered by double-stranded RNA (dsRNA).

As is described in detail below, a modulator of the invention, HC-Pro is known to cause the suppression of RNA silencing of inverted repeat, amplicon, and sense transgenes in plants and that this suppression is correlated with a decrease in the levels of siRNA in the plants. Furthermore, it has been discovered that HC-Pro is also capable of causing at the same time in these plants increase in the levels of specific miRNAs therein.

Modulators of the invention include, but are not limited to, any protein that is capable modulating the levels of one or more miRNAs in a plant cell. Such proteins include, but are not limited to eukaryotic and viral proteins, including the plant protein rg-CaM. See, Anandalakshmi *et al.* (2000) *Science* 290(5489):142-1444. Such modulators include, but are not limited to, viral proteins that are known to act as suppressors of gene silencing in plants (viral suppressors). Such viral suppressors may be selected from the 5'-proximal region of potyviral genomes, such as from tobacco etch potyviral (TEV), potato virus Y and the like. The skilled artisan will appreciate that the 5' proximal regions are also termed P1/HC-Pro sequence. The P1/HC-Pro sequence has been shown to interfere with a general antiviral system in plants, thereby permitting viruses to accumulate beyond the normal host-mediated limits. The sequence may act as a suppressor of transgene-induced, amplicon induced and virus-induced gene silencing. Other modulators or viral suppressors include the 2b protein of cucumber mosaic virus (CMV), HC-Pro of potato virus Y (PVY), other virally encoded proteins, and the like. It is recognized that sequences from other organisms, including for example, other plants, animals, and viruses, may also act as modulators. Such modulators may be assayed for the activity of modulating the levels of one or more miRNAs in a plant as described below for HC-Pro. Such modulators may also be assayed for the suppression of gene silencing by the methods described below and in Brigneti *et al.* (1998) *EMBO Journal* 17:6739-6746, herein incorporated by reference. These methods utilize plants exhibiting post-transcriptional gene silencing of a marker gene, such as, for example, green fluorescent protein. Such plants are infected with a potyvirus or with CMV. Silenced plants were also infected with potato virus X (PVX) and with chimeric constructs carrying coding sequences from PVY and CMV. Expression of sequences comprising a modulator would

result in transgene expression of the marker protein. Additionally, the modulators will cause an increase in the level of one or more miRNAs in the plant.

Variants or sequences having substantial identity or homology with the modulator molecules may be utilized in the practices of the invention. That is, the modulator may
 5 be modified yet still retain the ability to modulate the level of one or more miRNAs in a plant or plant cell, and, in certain embodiments of the invention, the modulator may also retain the ability to act as a suppressor of post-transcriptional gene silencing. Generally, the modulator will have at least about 40%, 50%, 60%, 65%, 70%, generally at least about 75%, 80%, 85%, preferably at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%,
 10 97%, and more preferably at least about 98%, 99% or more sequence identity to the native modulator sequence as determined by sequence alignment programs described elsewhere herein using default parameters.

Sequence relationships between two or more nucleic acids or polynucleotides are generally defined as sequence identity, percentage of sequence identity, and substantial
 15 identity. See, for example, "Pedestrian Guide to Analyzing Sequence Data Bases" at www.embl-heidelberg.de/~schneide/paper/springer96/springer.html. In determining sequence identity, a "reference sequence" is used as a basis for sequence comparison. The reference sequence may be a subset or the entirety of a specified sequence. That is, the reference sequence may be a full-length gene sequence or a segment of the gene
 20 sequence.

Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent identity between any two sequences can be accomplished using a mathematical algorithm. Non-limiting examples of such mathematical algorithms are the algorithm of Myers and Miller (1988) *CABIOS* 4:11-17;
 25 the local homology algorithm of Smith *et al.* (1981) *Adv. Appl. Math.* 2:482; the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443-453; the search-for-similarity-method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci.* 85:2444-2448; the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 87:2264, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA*
 30 90:5873-5877.

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, California); the ALIGN program (Version 2.0) and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Version 8 (available from Genetics Computer Group (GCG), 575 Science Drive, Madison, Wisconsin, USA). Alignments using these programs can be performed using the default parameters. The CLUSTAL program is well described by Higgins *et al.* (1988) *Gene* 73:237-244 (1988); Higgins *et al.* (1989) *CABIOS* 5:151-153; Corpet *et al.* (1988) *Nucleic Acids Res.* 16:10881-90; Huang *et al.* (1992) *CABIOS* 8:155-65; and Pearson *et al.* (1994) *Meth. Mol. Biol.* 24:307-331. The ALIGN program is based on the algorithm of Myers and Miller (1988) *supra*. A PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used with the ALIGN program when comparing amino acid sequences. The BLAST programs of Altschul *et al.* (1990) *J. Mol. Biol.* 215:403 are based on the algorithm of Karlin and Altschul (1990) *supra*. BLAST nucleotide searches can be performed with the BLASTN program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleotide sequence encoding a protein of the invention. BLAST protein searches can be performed with the BLASTX program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to a protein or polypeptide of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res.* 25:3389. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. See Altschul *et al.* (1997) *supra*. When utilizing BLAST, Gapped BLAST, PSI-BLAST, the default parameters of the respective programs (e.g., BLASTN for nucleotide sequences, BLASTX for proteins) can be used. See <http://www.ncbi.nlm.nih.gov>. Alignment may also be performed manually by inspection.

Unless otherwise stated, sequence identity/similarity values provided herein refer to the value obtained using GAP Version 10 using the following parameters: % identity using GAP Weight of 50 and Length Weight of 3; % similarity using Gap Weight of 12 and Length Weight of 4, or any equivalent program. By "equivalent program" is

intended any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by the preferred program.

5 GAP uses the algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443-453, to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. GAP considers all possible alignments and gap positions and creates the alignment with the largest number of matched bases and the fewest gaps. It allows for the provision of a gap creation penalty and a gap extension
10 penalty in units of matched bases. GAP must make a profit of gap creation penalty number of matches for each gap it inserts. If a gap extension penalty greater than zero is chosen, GAP must, in addition, make a profit for each gap inserted of the length of the gap times the gap extension penalty. Default gap creation penalty values and gap
15 extension penalty values in Version 10 of the Wisconsin Genetics Software Package for protein sequences are 8 and 2, respectively. For nucleotide sequences the default gap creation penalty is 50 while the default gap extension penalty is 3. The gap creation and gap extension penalties can be expressed as an integer selected from the group of integers consisting of from 0 to 200. Thus, for example, the gap creation and gap extension
20 penalties can be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 or greater.

 GAP presents one member of the family of best alignments. There may be many members of this family, but no other member has a better quality. GAP displays four figures of merit for alignments: Quality, Ratio, Identity, and Similarity. The Quality is the metric maximized in order to align the sequences. Ratio is the quality divided by the
25 number of bases in the shorter segment. Percent Identity is the percent of the symbols that actually match. Percent Similarity is the percent of the symbols that are similar. Symbols that are across from gaps are ignored. A similarity is scored when the scoring matrix value for a pair of symbols is greater than or equal to 0.50, the similarity threshold. The scoring matrix used in Version 10 of the Wisconsin Genetics Software
30 Package is BLOSUM62 (see Henikoff and Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915).

"Sequence identity" or "identity" in the context of nucleic acid or polypeptide sequences refers to the nucleic acid bases or residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window.

"Percentage of sequence identity" refers to the value determined by comparing two

5 optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions as compared to the reference window for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of
10 matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

Polynucleotide sequences having "substantial identity" are those sequences having at least about 50%, 60% sequence identity, generally 70% sequence identity,
15 preferably at least 80%, more preferably at least 90%, and most preferably at least 95%, compared to a reference sequence using one of the alignment programs described above. Preferably sequence identity is determined using the default parameters determined by the program. Substantial identity of amino acid sequences generally means sequence identity of at least 50%, more preferably at least 70%, 80%, 90%, and most preferably at
20 least 95%.

Nucleotide sequences are generally substantially identical if the two molecules hybridize to each other under stringent conditions. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point for the specific sequence at a defined ionic strength and pH. Nucleic acid molecules that do not hybridize to each
25 other under stringent conditions may still be substantially identical if the polypeptides they encode are substantially identical. This occurs when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

As noted, hybridization of sequences may be carried out under stringent conditions. By "stringent conditions" is intended conditions under which a probe will
30 hybridize to its target sequence to a detectably greater degree than to other sequences. Stringent conditions are sequence-dependent and will be different in different

circumstances. Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g.,
5 greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1.0 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20X SSC= 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. It is recognized that the temperature salt
10 and wash conditions may be altered to increase or decrease stringency conditions. The duration of hybridization is generally less than about 24 hours, usually about 4 to about 12 hours. For the post-hybridization washes, the critical factors are the ionic strength and temperature of the final wash solution. See, Meinkoth and Wahl (1984) *Anal. Biochem.* 138:267-284.

15 As indicated, fragments and variants of the nucleotide sequences of the invention are encompassed herein. By "fragment" is intended a portion of the nucleotide sequence. Fragments of the modulator sequence will generally retain the biological activity of the native suppressor protein. Alternatively, fragments of the targeting sequence may or may not retain biological activity. Such targeting sequences may be useful as hybridization
20 probes, as antisense constructs, or as co-suppression sequences. Thus, fragments of a nucleotide sequence may range from at least about 20 nucleotides, about 50 nucleotides, about 100 nucleotides, and up to the full-length nucleotide sequence of the invention.

By "variants" is intended substantially similar sequences. For nucleotide sequences, conservative variants include those sequences that, because of the degeneracy
25 of the genetic code, encode the amino acid sequence of the modulator of the invention. Variant nucleotide sequences include synthetically derived sequences, such as those generated, for example, using site-directed mutagenesis. Generally, variants of a particular nucleotide sequence of the invention will have at least about 40%, 50%, 60%, 65%, 70%, generally at least about 75%, 80%, 85%, preferably at least about 90%, 91%,
30 92%, 93%, 94%, 95%, 96%, 97%, and more preferably at least about 98%, 99% or more

sequence identity to that particular nucleotide sequence as determined by sequence alignment programs described elsewhere herein using default parameters.

Variant modulator proteins may also be utilized. By "variant" protein is intended a protein derived from the native protein by deletion or addition of one or more amino acids to the N-terminal and/or C-terminal end of the native protein; deletion or addition of one or more amino acids at one or more sites in the native protein; or substitution of one or more amino acids at one or more sites in the native protein. Such variants may result from, for example, genetic polymorphism or human manipulation. Conservative amino acid substitutions will generally result in variants that retain biological function.

Variant proteins encompassed by the present invention are biologically active, that is they continue to possess the desired biological activity of the native protein, that is, modulator activity as described herein. Biologically active variants of a native modulator protein of the invention will have at least about 40%, 50%, 60%, 65%, 70%, generally at least about 75%, 80%, 85%, preferably at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, and more preferably at least about 98%, 99% or more sequence identity to the amino acid sequence for the native protein as determined by sequence alignment programs described elsewhere herein using default parameters. A biologically active variant of a protein of the invention may differ from that protein by as few as 1-15 amino acid residues, as few as 1-10, such as 6-10, as few as 5, as few as 4, 3, 2, or even 1 amino acid residue.

The target sequence also includes fragments and variants of proteins or regulator sequences. The target proteins of the invention may include those that are altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Methods for such manipulation are generally known in the art. See, for example, Kunkel (1985) *Proc. Natl. Acad. Sci. USA* 82:488-492; Kunkel *et al.* (1987) *Methods and Enzymol.* 154:367-382; and the references cited therein.

The methods of the invention are useful in any situation where increased or decreased expression of a nucleotide sequence is desired. Thus, the methods are useful for increasing or decreasing the expression of endogenous as well as exogenous sequences. For example, for exogenous sequences, the modulator sequence can be used to enhance expression of genes silenced using transgene-induced gene silencing. Therefore, the target sequence may be any nucleotide sequence of interest. In one

embodiment, the methods can be used to produce peptides or proteins that cannot effectively be commercially produced by existing gene expression systems. For example, some proteins cannot be expressed in mammalian systems because the protein interferes with cell viability, cell proliferation, cellular differentiation, or protein assembly in
5 mammalian cells. Such proteins include, but are not limited to, retinoblastoma protein, p53, angiostatin, and leptin. Likewise, the methods of the invention can be used to produce mammalian regulatory proteins. Other sequences of interest include proteins, hormones, growth factors, cytokines, preferably insulin, growth hormone, particularly human growth hormone, interferon, particularly γ -interferon, β -glucocerebrosidase,
10 serum albumin, particularly human serum albumin, hemoglobin, collagen, etc. In such instances, generally, the modulator sequence will be operably linked to a constitutive promoter.

In another embodiment, the methods of the invention can be used to produce transgenic seed and seed products. In this manner, the nucleotide sequence encoding the
15 RNA precursor and/of the modulator can be operably linked with a seed-preferred, or endosperm promoter. Such seed proteins of interest include, but are not limited to, starches, storage proteins, proteins with enhanced nutritional value, specialty oils, carotenoids, etc.

Generally, the methods of the invention can be used to increase or decrease the
20 expression of any gene or sequence of interest including therapeutic or immunogenic peptides and proteins, nucleic acids for controlling gene expression, genes to reproduce enzymatic pathways for chemical synthesis, genes to shunt an enzymatic pathway for enhanced expression of a particular intermediate or final product, industrial processes, and the like.

It is recognized that the methods of the invention can be used for increased or
25 decreased expression of one, two or more target nucleotide sequences in transformed plants, plant cells and tissues, seed, and the like. Thus, in some embodiments, it may be beneficial to provide the methods in a plant culture system for production of metabolites, peptides or proteins of interest.

30 As discussed, a number of promoters can be used in the practice of the invention. The promoters can be selected based on the desired outcome. Generally, the RNA

precursor nucleotide sequence and the modulator sequences can be combined with promoters of choice to alter gene expression if the target sequences in the tissue or organ of choice. Thus, the RNA precursor nucleotide sequence or modulator nucleotide sequence can be combined with constitutive, tissue-preferred, inducible, developmental, or other promoters for expression in plants depending upon the desired outcome.

Constitutive promoters include, for example, CaMV 35S promoter (Odell *et al.* (1985) *Nature* 313:810-812); rice actin (McElroy *et al.* (1990) *Plant Cell* 2:163-171); ubiquitin (Christensen *et al.* (1989) *Plant Mol. Biol.* 12:619-632 and Christensen *et al.* (1992) *Plant Mol. Biol.* 18:675-689); pEMU (Last *et al.* (1991) *Theor. Appl. Genet.* 81:581-588); MAS (Velten *et al.* (1984) *EMBO J.* 3:2723-2730); ALS promoter (U.S. Application Serial No. 08/409,297), and the like. Other constitutive promoters include those in U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; and 5,608,142.

A number of inducible promoters are known in the art. For resistance genes, a pathogen-inducible promoter can be utilized. Such promoters include those from pathogenesis-related proteins (PR proteins), which are induced following infection by a pathogen; e.g., PR proteins, SAR proteins, beta-1,3-glucanase, chitinase, etc. See, for example, Redolfi *et al.* (1983) *Neth. J. Plant Pathol.* 89:245-254; Uknes *et al.* (1992) *Plant Cell* 4:645-656; and Van Loon (1985) *Plant Mol. Virol.* 4:111-116. Of particular interest are promoters that are expressed locally at or near the site of pathogen infection. See, for example, Marineau *et al.* (1987) *Plant Mol. Biol.* 9:335-342; Matton *et al.* (1989) *Molecular Plant-Microbe Interactions* 2:325-331; Somsisch *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:2427-2430; Somsisch *et al.* (1988) *Mol. Gen. Genet.* 2:93-98; and Yang (1996) *Proc. Natl. Acad. Sci. USA* 93:14972-14977. See also, Chen *et al.* (1996) *Plant J.* 10:955-966; Zhang *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:2507-2511; Warner *et al.* (1993) *Plant J.* 3:191-201; Siebertz *et al.* (1989) *Plant Cell* 1:961-968; U.S. Patent No. 5,750,386; Cordero *et al.* (1992) *Physiol. Mol. Plant Path.* 41:189-200; and the references cited therein.

Additionally, as pathogens find entry into plants through wounds or insect damage, a wound-inducible promoter may be used in the DNA constructs of the invention. Such wound-inducible promoters include potato proteinase inhibitor (pin II)

gene (Ryan (1990) *Ann. Rev. Phytopath.* 28:425-449; Duan *et al.* (1996) *Nature Biotechnology* 14:494-498); *wun1* and *wun2*, US Patent No. 5,428,148; *win1* and *win2* (Stanford *et al.* (1989) *Mol. Gen. Genet.* 215:200-208); *systemin* (McGurl *et al.* (1992) *Science* 225:1570-1573); *WIP1* (Rohmeier *et al.* (1993) *Plant Mol. Biol.* 22:783-792; 5 Eckelkamp *et al.* (1993) *FEBS Letters* 323:73-76); *MPI* gene (Corderok *et al.* (1994) *Plant J.* 6(2):141-150); and the like. Such references are herein incorporated by reference.

Chemical-regulated promoters can be used to modulate the expression of a gene in a plant through the application of an exogenous chemical regulator. Depending upon 10 the objective, the promoter may be a chemical-inducible promoter, where application of the chemical induces gene expression, or a chemical-repressible promoter, where application of the chemical represses gene expression. Chemical-inducible promoters are known in the art and include, but are not limited to, the maize *In2-2* promoter, which is activated by benzenesulfonamide herbicide safeners, the maize *GST* promoter, which is 15 activated by hydrophobic electrophilic compounds that are used as pre-emergent herbicides, and the tobacco *PR-1a* promoter, which is activated by salicylic acid. Other chemical-regulated promoters of interest include steroid-responsive promoters (see, for example, the glucocorticoid-inducible promoter in Schena *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:10421-10425 and McNellis *et al.* (1998) *Plant J.* 14(2):247-257) and 20 tetracycline-inducible and tetracycline-repressible promoters (see, for example, Gatz *et al.* (1991) *Mol. Gen. Genet.* 227:229-237, and U.S. Patent Nos. 5,814,618 and 5,789,156), herein incorporated by reference.

Where enhanced expression in particular tissues is desired, tissue-preferred promoters can be utilized. Tissue-preferred promoters include those described by 25 Yamamoto *et al.* (1997) *Plant J.* 12(2):255-265; Kawamata *et al.* (1997) *Plant Cell Physiol.* 38(7):792-803; Hansen *et al.* (1997) *Mol. Gen. Genet.* 254(3):337-343; Russell *et al.* (1997) *Transgenic Res.* 6(2):157-168; Rinehart *et al.* (1996) *Plant Physiol.* 112(3):1331-1341; Van Camp *et al.* (1996) *Plant Physiol.* 112(2):525-535; Canevascini *et al.* (1996) *Plant Physiol.* 112(2):513-524; Yamamoto *et al.* (1994) *Plant Cell Physiol.* 30 35(5):773-778; Lam (1994) *Results Probl. Cell Differ.* 20:181-196; Orozco *et al.* (1993)

Plant Mol Biol. 23(6):1129-1138; Matsuoka *et al.* (1993) *Proc Natl. Acad. Sci. USA* 90(20):9586-9590; and Guevara-Garcia *et al.* (1993) *Plant J.* 4(3):495-505.

Leaf-preferred promoters include, Yamamoto *et al.* (1997) *Plant J.* 12(2):255-265; Kwon *et al.* (1994) *Plant Physiol.* 105:357-67; Yamamoto *et al.* (1994) *Plant Cell Physiol.* 35(5):773-778; Gotor *et al.* (1993) *Plant J* 3:509-18; Orozco *et al.* (1993) *Plant Mol. Biol.* 23(6):1129-1138; and Matsuoka *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90(20):9586-9590.

Root-preferred promoters are known and can be selected from the many available from the literature. See, for example, Hire *et al.* (1992) *Plant Mol. Biol.* 20(2): 207-218 (soybean root-preferred glutamine synthetase gene); Keller and Baumgartner (1991) *Plant Cell* 3(10):1051-1061 (root-preferred control element in the GRP 1.8 gene of French bean); Sanger *et al.* (1990) *Plant Mol. Biol.* 14(3):433-443 (root-preferred promoter of the mannopine synthase (MAS) gene of *Agrobacterium tumefaciens*); Miao *et al.* (1991) *Plant Cell* 3(1):11-22 (full-length cDNA clone encoding cytosolic glutamine synthetase (GS), which is expressed in roots and root nodules of soybean). Bogusz *et al.* (1990) *Plant Cell* 2(7):633-641 (root-preferred promoters from hemoglobin genes from the nitrogen-fixing nonlegume *Parasponia andersonii* and the related non-nitrogen-fixing nonlegume *Trema tomentosa*). Leach and Aoyagi (1991) *Plant Science* (Limerick) 79(1):69-76 (rolC and rolD root-inducing genes of *Agrobacterium rhizogenes*); Teeri *et al.* (1989) *EMBO J.* 8(2):343-350 (octopine synthase and TR2' gene); (VfENOD-GRP3 gene promoter); Kuster *et al.* (1995) *Plant Mol. Biol.* 29(4):759-772 and Capana *et al.* (1994) *Plant Mol. Biol.* 25(4):681-691 rolB promoter. See also U.S. Patent Nos. 5,837,876; 5,750,386; 5,633,363; 5,459,252; 5,401,836; 5,110,732; and 5,023,179.

Anther or pollen-preferred promoters may be used to create male sterile plants. While either the RNA precursor nucleotide sequence or the modulator nucleotide sequence may be operably linked to such promoters, it may be preferred to express both the modulator and the targeting sequence with an anther preferred or pollen preferred promoter to prevent even low expression of the toxin in other tissues of the plant.

"Seed-preferred" promoters include both "seed-specific" promoters (those promoters active during seed development such as promoters of seed storage proteins) as well as "seed-germinating" promoters (those promoters active during seed germination).

See Thompson *et al.* (1989) *BioEssays* 10:108, herein incorporated by reference. Such seed-preferred promoters include, but are not limited to, Cim1 (cytokinin-induced message); cZ19B1 (maize 19 kDa zein); celA (cellulose synthase); gama-zein; Glob-1; bean β -phascolin; napin; β -conglycinin; soybean lectin; cruciferin; maize 15 kDa zein; 22
 5 kDa zein; 27 kDa zein; g-zein; waxy; shrunken 1; shrunken 2; globulin 1, etc.

The RNA precursor and modulator sequences of the invention may be provided in nucleotide sequence constructs or expression cassettes for expression in the plant of interest. The cassette will include 5' and 3' regulatory sequences operably linked to an miRNA nucleotide sequence or modulator nucleotide sequence sequence of the
 10 invention. By "operably linked" is intended a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence. Generally, operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading frame.

15 The expression cassette may additionally contain at least one additional gene to be cotransformed into the organism. Alternatively, the additional gene(s) can be provided on multiple expression cassettes.

Such an expression cassette is provided with a plurality of restriction sites for insertion of the sequences of the invention to be under the transcriptional regulation of
 20 the regulatory regions. The expression cassette may additionally contain selectable marker genes.

The expression cassette will include in the 5'-3' direction of transcription, a transcriptional and translational initiation region, a DNA sequence of the invention, and a transcriptional and translational termination region functional in plants. The
 25 transcriptional initiation region, the promoter, may be native or analogous or foreign or heterologous to the plant host. Additionally, the promoter may be the natural sequence or alternatively a synthetic sequence. By "foreign" is intended that the transcriptional initiation region is not found in the native plant into which the transcriptional initiation region is introduced. As used herein, a chimeric gene comprises a coding sequence
 30 operably linked to a transcription initiation region that is heterologous to the coding sequence.

The termination region may be native with the transcriptional initiation region, may be native with the operably linked DNA sequence of interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also Guerineau *et al.* (1991) *Mol. Gen. Genet.* 262:141-144; Proudfoot (1991) *Cell* 64:671-674; Sanfacon *et al.* (1991) *Genes Dev.* 5:141-149; Mogen *et al.* (1990) *Plant Cell* 2:1261-1272; Munroe *et al.* (1990) *Gene* 91:151-158; Ballas *et al.* (1989) *Nucleic Acids Res.* 17:7891-7903; and Joshi *et al.* (1987) *Nucleic Acid Res.* 15:9627-9639.

Where appropriate, the gene(s) for enhanced expression may be optimized for expression in the transformed plant. That is, the genes can be synthesized using plant-preferred codons corresponding to the plant of interest. Methods are available in the art for synthesizing plant-preferred genes. See, for example, U.S. Patent Nos. 5,380,831, and 5,436,391, and Murray *et al.* (1989) *Nucleic Acids Res.* 17:477-498, herein incorporated by reference.

Additional sequence modifications are known to enhance gene expression in a cellular host. These include elimination of sequences encoding spurious polyadenylation signals, exon-intron splice site signals, transposon-like repeats, and other such well-characterized sequences that may be deleterious to gene expression. The G-C content of the sequence may be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. When desired, the sequence is modified to avoid predicted hairpin secondary mRNA structures. However, it is recognized that in the case of nucleotide sequences encoding the miRNA precursors, one or more hairpin and other secondary structures may be desired for proper processing of the precursor into an mature miRNA and/or for the functional activity of the miRNA in gene silencing.

The expression cassettes may additionally contain 5' leader sequences in the expression cassette construct. Such leader sequences can act to enhance translation. Translation leaders are known in the art and include: picornavirus leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region) (Elroy-Stein *et al.* (1989) *PNAS USA* 86:6126-6130); potyvirus leaders, for example, TEV leader (Tobacco Etch

Virus) (Allison *et al.* (1986); MDMV leader (Maize Dwarf Mosaic Virus); *Virology* 154:9-20), and human immunoglobulin heavy-chain binding protein (BiP), (Macejak *et al.* (1991) *Nature* 353:90-94); untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4) (Jobling *et al.* (1987) *Nature* 325:622-625); tobacco mosaic virus leader (TMV) (Gallie *et al.* (1989) in *Molecular Biology of RNA*, ed. Cech (Liss, New York), pp. 237-256); and maize chlorotic mottle virus leader (MCMV) (Lommel *et al.* (1991) *Virology* 81:382-385). See also, Della-Cioppa *et al.* (1987) *Plant Physiol.* 84:965-968.

In preparing the expression cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, e.g., transitions and transversions, may be involved.

The invention involves plant cells and plants that comprise two or more nucleotide sequence constructs. Any means for producing a plant comprising the nucleotide sequence constructs described herein are encompassed by the present invention. For example, a nucleotide sequence encoding the modulator can be used to transform a plant at the same time as the nucleotide sequence encoding the precursor RNA. The nucleotide sequence encoding the precursor mRNA can be introduced into a plant that has already been transformed with the modulator nucleotide sequence. Alternatively, transformed plants, one expressing the modulator and one expressing the RNA precursor, can be crossed to bring the genes together in the same plant. Likewise, viral vectors may be used to express gene products by various methods generally known in the art. Suitable plant viral vectors for expressing genes should be self-replicating, capable of systemic infection in a host, and stable. Additionally, the viruses should be capable of containing the nucleic acid sequences that are foreign to the native virus forming the vector. Transient expression systems may also be used.

Plants transformed with a nucleotide sequence construct of the invention may be produced by standard techniques known in the art for the genetic manipulation of plants.

DNA can be transformed into plant cells using any suitable technology, such as a disarmed Ti-plasmid vector carried by *Agrobacterium* exploiting its natural gene transferability (EP-A-270355, EP-A-0116718, *NAR* 12(22):8711-87215 (1984), Townsend *et al.*, U.S. Patent No. 5,563,055); particle or microprojectile bombardment

5 (U.S. Patent No. 5,100,792, EP-A-444882, EP-A-434616; Sanford *et al.*, U.S. Patent No. 4,945,050; Tomes *et al.* (1995) "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg and Phillips (Springer-Verlag, Berlin); and McCabe *et al.* (1988) *Biotechnology* 6:923-926); microinjection (WO 92/09696, WO 94/00583, EP 331083, EP

10 175966, Green *et al.* (1987) *Plant Tissue and Cell Culture*, Academic Press, Crossway *et al.* (1986) *Biotechniques* 4:320-334); electroporation (EP 290395, WO 8706614, Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:5602-5606; D'Halluin *et al.* (1992) *Plant Cell* 4:1495-1505) other forms of direct DNA uptake (DE 4005152, WO 9012096, U.S. Patent No. 4,684,611, Paszkowski *et al.* (1984) *EMBO J.* 3:2717-2722); liposome-mediated

15 DNA uptake (e.g., Freeman *et al.* (1984) *Plant Cell Physiol.* 29:1353); or the vortexing method (e.g., Kindle (1990) *Proc. Nat. Acad. Sci. U.S.A.* 87:1228). Physical methods for the transformation of plant cells are reviewed in Oard (1991) *Biotech. Adv.* 9:1-11. See generally, Weissinger *et al.* (1988) *Ann. Rev. Genet.* 22:421-477; Sanford *et al.* (1987) *Particulate Science and Technology* 5:27-37; Christou *et al.* (1988) *Plant Physiol.*

20 87:671-674; McCabe *et al.* (1988) *Bio/Technology* 6:923-926; Finer and McMullen (1991) *In Vitro Cell Dev. Biol.* 27P:175-182; Singh *et al.* (1998) *Theor. Appl. Genet.* 96:319-324; Datta *et al.* (1990) *Biotechnology* 8:736-740; Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:4305-4309; Klein *et al.* (1988) *Biotechnology* 6:559-563; Tomes, U.S. Patent No. 5,240,855; Buising *et al.*, U.S. Patent Nos. 5,322,783 and 5,324,646; Klein *et al.* (1988) *Plant Physiol.* 91:440-444; Fromm *et al.* (1990) *Biotechnology* 8:833-839;

25 Hooykaas-Van Slogteren *et al.* (1984) *Nature (London)* 311:763-764; Bytebier *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:5345-5349; De Wet *et al.* (1985) in *The Experimental Manipulation of Ovule Tissues*, ed. Chapman *et al.* (Longman, New York), pp. 197-209; Kaeppeler *et al.* (1990) *Plant Cell Reports* 9:415-418 and Kaeppeler *et al.*

30 (1992) *Theor. Appl. Genet.* 84:560-566; Li *et al.* (1993) *Plant Cell Reports* 12:250-255

and Christou and Ford (1995) *Annals of Botany* 75:407-413; Osjoda *et al.* (1996) *Nature Biotechnology* 14:745-750; all of which are herein incorporated by reference.

- Agrobacterium* transformation is widely used by those skilled in the art to transform dicotyledonous species. Recently, there has been substantial progress towards the routine production of stable, fertile transgenic plants in almost all economically relevant monocot plants (Toriyama *et al.* (1988) *Bio/Technology* 6:1072-1074; Zhang, *et al.* (1988) *Plant Cell Rep.* 7:379-384; Zhang *et al.* (1988) *Theor. Appl. Genet.* 76:835-840; Shimamoto *et al.* (1989) *Nature* 338:274-276; Datta *et al.* (1990) *Bio/Technology* 8:736-740; Christou *et al.* (1991) *Bio/Technology* 9:957-962; Peng *et al.* (1991) International Rice Research Institute, Manila, Philippines, pp. 563-574; Cao *et al.* (1992) *Plant Cell Rep.* 11:585-591; Li *et al.* (1993) *Plant Cell Rep.* 12:250-255; Rathore *et al.* (1993) *Plant Mol. Biol.* 21:871-884; Fromm *et al.* (1990) *Bio/Technology* 8:833-839; Tomes *et al.* (1995) "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg and Phillips (Springer-Verlag, Berlin); D'Halluin *et al.* (1992) *Plant Cell* 4:1495-1505; Walters *et al.* (1992) *Plant Mol. Biol.* 18:189-200; Koziel *et al.* (1993) *Biotechnology* 11:194-200; Vasil, I. K. (1994) *Plant Mol. Biol.* 25:925-937; Weeks *et al.* (1993) *Plant Physiol.* 102:1077-1084; Somers *et al.* (1992) *Bio/Technology* 10:1589-1594; WO 92/14828). In particular, *Agrobacterium* mediated transformation is now emerging also as an highly efficient transformation method in monocots (Hiei, *et al.* (1994) *The Plant Journal* 6:271-282). See also, Shimamoto, K. (1994) *Current Opinion in Biotechnology* 5:158-162; Vasil, *et al.* (1992) *Bio/Technology* 10:667-674; Vain, *et al.* (1995) *Biotechnology Advances* 13(4):653-671; Vasil, *et al.* (1996) *Nature Biotechnology* 14:702).
- Microprojectile bombardment, electroporation and direct DNA uptake are preferred where *Agrobacterium* is inefficient or ineffective. Alternatively, a combination of different techniques may be employed to enhance the efficiency of the transformation process, e.g., bombardment with *Agrobacterium*-coated microparticles (EP-A-486234) or microprojectile bombardment to induce wounding followed by co-cultivation with *Agrobacterium* (EP-A-486233).

Following transformation, a plant may be regenerated, e.g., from single cells, callus tissue or leaf discs, as is standard in the art. Almost any plant can be entirely regenerated from cells, tissues, and organs of the plant. Available techniques are reviewed in Vasil *et al.* (1984) in *Cell Culture and Somatic Cell Genetics of Plants, Vols. I, II, and III, Laboratory Procedures and Their Applications* (Academic Press); and Weissbach *et al.* (1989) *Methods For Plant Mol. Biol.*

The transformed plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrid having expression of the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that expression of the desired phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure expression of the desired phenotypic characteristic has been achieved.

The particular choice of a transformation technology will be determined by its efficiency to transform certain plant species as well as the experience and preference of the person practicing the invention with a particular methodology of choice. It will be apparent to the skilled person that the particular choice of a transformation system to introduce nucleic acid into plant cells is not essential to or a limitation of the invention, nor is the choice of technique for plant regeneration.

Also according to the invention there is provided a plant cell having the nucleotide sequence constructs of the invention. A further aspect of the present invention provides a method of making such a plant cell involving introduction of a vector including the construct into a plant cell. For integration of the construct into the plant genome, such introduction will be followed by recombination between the vector and the plant cell genome to introduce the sequence of nucleotides into the genome. RNA encoded by the introduced nucleic acid construct may then be transcribed in the cell and descendants thereof, including cells in plants regenerated from transformed material. A gene stably incorporated into the genome of a plant is passed from generation to generation to descendants of the plant, so such descendants should show the desired phenotype.

The present invention also provides a plant comprising a plant cell as disclosed. Transformed seeds and plant parts are also encompassed.

In addition to a plant, the present invention provides any clone of such a plant, seed, selfed or hybrid progeny and descendants, and any part of any of these, such as cuttings, seed. The invention provides any plant propagule, that is any part which may be used in reproduction or propagation, sexual or asexual, including cuttings, seed and so on. Also encompassed by the invention is a plant which is a sexually or asexually propagated off-spring, clone or descendant of such a plant, or any part or propagule of said plant, off-spring, clone or descendant. Plant extracts and derivatives are also provided.

The present invention may particularly be applied in plants such as natural hosts of a plant virus, including any mentioned herein, though it is an advantage of embodiments of the present invention that viruses may be used for gene silencing in plants which are not their natural hosts.

The present invention may be used for transformation of any plant species, including, but not limited to, corn (*Zea mays*), canola (*Brassica napus*, *Brassica rapa* ssp.), alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor*, *Sorghum vulgare*), sunflower (*Helianthus annuus*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*, *Nicotiana benthamiana*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium hirsutum*), sweet potato (*Ipomoea batatas*), cassava (*Manihot esculenta*), coffee (*Coffea* spp.), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), oats, barley, *Arabidopsis* spp., vegetables, ornamentals, and conifers.

Preferably, plants of the present invention are crop plants (for example, cereals and pulses, maize, wheat, potatoes, tapioca, rice, sorghum, millet, cassava, barley, pea, and other root, tuber, or seed crops. Important seed crops are oil-seed rape, sugar beet, maize, sunflower, soybean, and sorghum. Horticultural plants to which the present invention may be applied may include lettuce, endive, and vegetable brassicas including cabbage, broccoli, and cauliflower, and carnations and geraniums. The present invention may be

applied to tobacco, cucurbits, carrot, strawberry, sunflower, tomato, pepper, chrysanthemum, poplar, eucalyptus, and pine.

Grain plants that provide seeds of interest include oil-seed plants and leguminous plants. Seeds of interest include grain seeds, such as corn, wheat, barley, rice, sorghum, rye, etc. Oil seed plants include cotton, soybean, safflower, sunflower, *Brassica*, maize, alfalfa, palm, coconut, etc. Leguminous plants include beans and peas. Beans include guar, locust bean, fenugreek, soybean, garden beans, cowpea, mungbean, lima bean, fava bean, lentils, chickpea, etc.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

EXAMPLE 1

HC-Pro suppression of IR- or amplicon-induced RNA silencing interferes with the accumulation of siRNAs and promotes accumulation of a novel class of slightly larger RNAs

RNA silencing is induced by dsRNA that is cleaved into the siRNAs that mediate sequence-specific RNA degradation. It had been previously shown that HC-Pro suppression of RNA silencing induced by the b-glucuronidase (GUS) sense transgene eliminates the accumulation of siRNAs (Figure 1A, line 6b5 and Figure 1B, lanes 4 and 5; Elmayan and Vaucheret, 1996; Mallory *et al.*, 2001). To determine if HC-Pro suppression of RNA silencing in IR and amplicon transgenic lines T4 and 155 (Angell and Baulcombe, 1997; Hobbs *et al.*, 1990) also eliminates siRNA accumulation, small RNAs were analyzed from the offspring of crosses between these lines and either a non-transformed (NT) control plant or an HC-Pro expressing line (line X-27-8; Mallory *et al.*, 2001). The IR transgene in line T4 contains two GUS genes arranged as an inverted repeat (Figure 1A) and therefore produces GUS dsRNA via read-through transcription. The amplicon transgene in line 155 is comprised of complementary DNA encoding the

potato virus X (PVX) genomic RNA in which the coat protein gene is replaced by the GUS locus (Figure 1A). The transcript produced by the amplicon transgene is a replicating PVX vector and thus produces dsRNA replication intermediates with homology to both PVX and GUS sequences.

5 As expected, the IR- and amplicon-silenced lines, like the sense transgene-silenced line 6b5, accumulated siRNAs. The small RNAs produced in these three different lines were analyzed by high-resolution gel electrophoresis. In each case, the size of the small RNAs was very similar, ranging from 21-24 nt and comprised of two major populations, one corresponding to approximately 22 nt and the other to approximately 24
10 nt (Figure 1B, lanes 1, 4 and 6; lane 1 is a longer exposure of lane 2). The small RNAs in each of these silenced lines hybridized to probes specific to both sense-strand GUS RNA (Figure 1B) and antisense-strand GUS RNA (Figure 1C), indicating the double stranded nature expected for siRNAs. Thus, these small RNAs have all the characteristics of bonafide siRNAs.

15 Examination of small RNAs isolated from sense, IR and amplicon transgene lines crossed with a line expressing HC-Pro showed that HC-Pro interfered with accumulation of the 21-24 nt siRNAs in all three of these lines (Figure 1B, lanes 3, 5, and 7; lane 9 is a shorter exposure of lane 7). However, surprisingly, HC-Pro suppression of IR- and amplicon-induced RNA silencing resulted in accumulation of a longer species of small
20 RNAs, approximately 25-27 nt (Figure 1B, lanes 3 and 7). These 25-27 nt small RNAs accumulated to very high levels during HC-Pro suppression of amplicon-induced RNA silencing (Figure 1B, lane 7), presumably reflecting the vastly increased amount of replicating PVX-GUS RNA in this line (Figure 2A, lane 3 versus Figure 2B, lane 4; Mallory *et al.*, 2002). Like the RNA silencing-associated small RNAs, the double
25 stranded nature of the longer small RNAs was evidenced by hybridization with both sense-strand (Figure 1B) and antisense-strand GUS RNA probes (Figure 1C). Thus, suppression of RNA silencing by HC-Pro eliminates siRNAs for all three types of transgenes, but in the case of IR and amplicon transgenes, a larger class of small RNAs accumulates instead. This result suggests that HC-Pro either interferes with the
30 accumulation or processing of the double stranded small RNA precursors or alters the stability of the small RNA products.

EXAMPLE 2

HC-Pro increases the level of miRNA accumulation in tobacco

5 HC-Pro inhibits siRNA accumulation (Figure 1), suggesting that HC-Pro may alter the activity of the Dicer-like RNase III responsible for siRNA production. Because miRNAs are also products of a Dicer-like RNase III in plants (Reinhart *et al.*, 2002), we examined the effect of HC-Pro on miRNA accumulation. Four miRNAs were chosen for these experiments. Each was identified in Arabidopsis, but predicted, on the basis of
10 sequence similarity, to be highly conserved throughout the flowering plants. The expression of all four miRNAs was detected in tobacco supporting the phylogenetic conservation of the miRNAs (Reinhart *et al.*, 2002). Strikingly, we found that HC-Pro increased the accumulation of these miRNAs approximately 5 to 10 fold (Figure 3A, lanes 1-8). No miRNA precursors were detected in HC-Pro-expressing or nonexpressing
15 lines suggesting that they are rapidly processed, degraded or too large to be resolved on these gels. The increase in miRNA accumulation was independent of the silencing locus present in the plants because there was little difference in miRNA levels in 155 X HC-Pro, 6b5 X HC-Pro and T4 X HC-Pro plant lines (data not shown). Furthermore, the level of miRNA accumulation was similar in a GUS-expressing control line and a GUS-
20 silenced line (Figure 3A, lanes 9 and 10), suggesting that RNA silencing does not reduce miRNA accumulation by competing for components shared by the two pathways.

We also analyzed the effect of HC-Pro on accumulation of two of the miRNAs in flower, leaf and stem tissues. HC-Pro increased the accumulation of both miRNAs, and in some cases the increase was tissue specific (Figure 3B and C). For example, HC-Pro
25 preferentially enhances the level of miR167 accumulation in leaves as compared to stems or flowers. These data indicate that HC-Pro differentially increases the accumulation of some miRNAs in a tissue specific manner.

EXAMPLE 3

Construction and Optimization of Nucleotide sequences Encoding miRNAs

Precursors miRNA are designed to target specific messenger RNAs (mRNAs) and
 5 alter expression of the protein encoded by that mRNA by the method described
 hereinbelow or by any other method known in the art. The overall approach is to design
 an RNA transcript (the miRNA precursor) that is capable of being processed *via* the
 miRNA biogenesis pathway to produce an miRNA that is complementary to a particular
 target mRNA. The miRNA that is processed from this designer miRNA precursor is
 10 capable of binding to the targeted mRNA and altering the expression of the protein it
 encodes. The expression of the targeted protein is assayed to determine if expression of
 the miRNA precursor results in altered gene expression. There are three steps in
 implementation of the method: 1) the designer miRNA precursor is constructed and
 cloned into a binary vector 2) the designer miRNA is expressed in plants or plant tissues
 15 that also express the target mRNA 3) the expression of the protein encoded by the
 targeted mRNA is assayed. In one embodiment of the invention, the target mRNA
 encodes the reporter enzyme β -glucuronidase (GUS) and is constitutively expressed in
 transgenic *Nicotiana benthamiana* plants (for transient expression assays) or *Arabidopsis*
thaliana plants (for stable transformation assays). A more detailed description of the
 20 approach follows.

Step 1. Design of the miRNA precursor. The initial experiments will determine
 the features that are required in an miRNA precursor in order to achieve appropriate
 processing via the miRNA pathway. The initial miRNA precursors will be designed to
 mimic endogenous miRNA precursors such as those reported in Llave et al (2002) and
 25 Reinhart et al. (2002). These precursors are characteristically stem-loop structures in
 which the stem is not completely double-stranded. The single-stranded miRNA is
 processed from the stem of the precursor and is commonly flanked by bulges. In these
 studies, a number of miRNA precursors will be designed that have all the features of an
 endogenous miRNA precursor, but the position of the endogenous miRNA will be
 30 replaced with that of the desired miRNA (one that is complementary or partly
 complementary to the desired target mRNA). For example, the designer miRNA

precursor will contain a loop chosen from an endogenous miRNA precursor. The desired miRNA will be a sequence that is complementary to the GUS mRNA (initially the chosen miRNA sequence is complementary to either the 3'- untranslated region or the coding region, but limited to those regions) and will be inserted in a position analogous to an authentic miRNA within the precursor. Bulges will be included and these too will be taken from the context of the endogenous precursor.

Step 2. *Express the designer miRNA precursor in plant tissues that also express the target mRNA.* A number of such designer miRNA precursors will be constructed and cloned into a binary vector under control of a plant promoter using standard cloning techniques. The binary vector containing the designer miRNA precursors will then be introduced into *Agrobacterium* strains suitable for both transient expression and stable transformation into plant cells. The *Agrobacterium*-mediated transient expression system as described by Johansen and Carrington (2001) or Hamilton et al. (2002), both of which are herein incorporated by references, will be used in preliminary experiments because it is fast and easy. In these assays, the *Agrobacterium* culture is pressure-infiltrated into leaf tissues where it is transiently expressed at high levels in a confined region called the "spot" or the "patch". We will use *Nicotiana benthamiana* expressing the targeted GUS mRNA for the Agro-infiltration experiments. Alternatively, we will transform GUS-expressing *Arabidopsis* transgenic plants to produce stable transformants that express both the GUS mRNA and the putative GUS miRNA precursor. Stable transformation techniques are well known in the field and are described above.

Step 3. *Assay expression of the targeted gene.* Designer miRNA precursors that effectively target the GUS mRNA are capable of altering the expression of the GUS enzyme. GUS enzyme activity will be measured using a standard fluorometric assay. If the miRNA precursor successfully targets the mRNA, a decrease in GUS activity will be observed. Alternatively, since the mechanisms by which miRNAs alter gene expression are not completely understood, it is conceivable that we will observe an increase in GUS activity.

EXAMPLE 4

miRNA precursor designed to target the GUS mRNA

In a first example of an miRNA precursor designed to target GUS, the backbone
 5 for the precursor is amplified by polymerase chain reaction (PCR) from *Arabidopsis*
thaliana genomic DNA using the sequence of the predicted precursor for an endogenous
 miRNA (miR167). The PCR product is isolated and cloned into the TA cloning vector.
 The cloned miRNA precursor backbone is further modified by standard cloning
 techniques to replace the miR167 sequence with the sequence "gcgtaagggt aatgcgaggt ac"
 10 which has complete complementarity to a 22 nucleotide region of nucleotides 9978-1000
 within the GUS coding region. The entire GUS coding region is 1811 nucleotides. The
 endogenous 167miRNA sequence is located within the miRNA 167 precursor in a region
 that forms a double-stranded stem. Therefore, the designer GUS miRNA precursor will
 be further modified to recreate this region of double-strandedness (so that the GUS
 15 miRNA sequence will be located in a double-stranded stem in the precursor).

This construct is cloned into the intermediate vector pRTL2 (Carrington et al.
 (1990) *EMBO J* 9:1347-53) and then transferred along with the cauliflower mosaic virus
 35S promoter and terminator sequences to the binary vector pFA482 (An, G. (1985)
Plant Physiol. 79:568-570). The binary vector will contain the GUS miRNA precursor
 20 under control of the constitutive 35S cauliflower mosaic virus promoter. The vectors are
 used to transform an *Arabidopsis thaliana* transgenic line that expresses the GUS gene
 also under control of the 35S promoter. See Anandalakshmi *et al.* (1998) *Proc Natl Acad*
Sci USA 95:13-79-084) The GUS mRNA in this transgenic plant is the target for the
 GUS miRNA. Transformants carrying both the GUS transgene and the GUS miRNA
 25 precursor are examined for GUS activity. GUS activity is decreased or eliminated in
 plants that express the GUS miRNA precursor. The vectors are also used to transform
 tobacco via the protocol of An, G. (2002) *Plant Molecular Biology Manual*, Gelvin and
 Schulerpoot, eds., Kluwer Academic Publishing, Boston, pp. A-3, 1-19.

In a second example, a GUS miRNA precursor is constructed as described in the
 30 above example, but the GUS sequence chosen for the miRNA is complementary to a
 different region of the GUS mRNA coding region. Plants carrying both the GUS

transgene and the second GUS miRNA precursor are observed to have increased GUS activity. Transgenic plants are prepared as described above.

In a third example, GUS miRNA precursor is constructed as described in example 1 above, but the GUS sequence chosen for the miRNA is not completely complementary to the GUS mRNA, containing a three base-pair mismatch in the approximate center of the miRNA. GUS activity in plants carrying both the GUS transgene and the GUS miRNA precursor is reduced or eliminated. Transgenic plants are prepared as described above.

In a fourth example, a GUS miRNA precursor is designed using the backbone from a different predicted endogenous miRNA precursor (miR171). This miRNA precursor backbone is PCR amplified and cloned exactly as described for the miR167 precursor described in example 1 and the miRNA 171 sequences replaced by the 21 nucleotides of sequence complementary to GUS exactly as in example 1. GUS activity in plants carrying both the GUS transgene and the GUS miRNA precursor based on the backbone of miR 171 is reduced or eliminated. Transgenic plants are prepared as described above.

EXAMPLE 5

An miRNA precursor designed to target *SULFUR* mRNA

20

An miRNA precursor is designed as described above, but using sequences completely complementary to 21 nucleotides within the coding region of the endogenous *SULFUR* gene encoding the protein magnesium cheletase. This gene is required for accumulation of chlorophyll in the plant. The *SULFUR* gene is an example of a reporter gene because, if the expression of the gene is eliminated, the plant will not be green. *Arabidopsis thaliana* plants are transformed with the *SULFUR* miRNA precursor and transformants carrying the *SULFUR* miRNA precursor are white. Vectors are constructed and plants are transformed as described in Example4.

The methods described hereinabove are not limited to use with the GUS gene, other reporter genes, or transgenes. The methods of the invention can be utilized with any other target nucleotide sequence in a plant, including both exogenous and

30

endogenous genes. Similarly, although initial techniques for expressing the designer miRNA precursors in the plant are *Agrobacterium*-mediated transient and stable transformation of *Nicotiana benthamiana* or *Arabidopsis thaliana* plants, the invention is not limited to these plants or these methods of bioengineering plants.

5

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All publications and patent applications mentioned in the specification are
 30 indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same

extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that
5 certain changes and modifications may be practiced within the scope of the appended claims.

THAT WHICH IS CLAIMED:

1. A method for modulating the expression of a first target sequence in a plant cell, said method comprising transforming said plant cell with an RNA precursor construct, said construct comprising a first promoter that drives expression in a plant cell operably linked to a first nucleotide sequence encoding a precursor RNA, said precursor having at least one miRNA sequence incorporated into the precursor RNA sequence, wherein said miRNA sequence is complementary to a portion of said first target sequence.

10

15

COMPOSITIONS AND METHODS FOR THE MODULATION
OF GENE EXPRESSION IN PLANTS

ABSTRACT

Compositions and methods for modulating nucleotide sequence expression,
5 particularly for modulating gene expression in plants, are provided. The compositions
comprise precursor RNA constructs for the expression of an RNA precursor. The
precursor RNA construct comprises a promoter that is expressed in a plant cell driving
the expression of a precursor RNA having a microRNA. The miRNA is complementary
or partially complementary to a portion of a target gene or nucleotide sequence and
10 function to modulate expression of the target sequence or gene. In this manner, the RNA
precursor construct can be designed to modulate expression of any nucleotide sequence
of interest, either an endogenous plant gene or alternatively a transgene. The precursor
RNA constructs may be used in combination with modulators to enhance the effect on
gene expression. Expression of a modulator in the presence of the precursor RNA alters
15 the accumulation of miRNAs and thus enhances the regulatory capabilities of miRNAs.
The invention further comprises the use of a modulator to control gene expression via
both the siRNA and the miRNA pathway. Transformed plants, tissues, cells and seeds
are also provided.

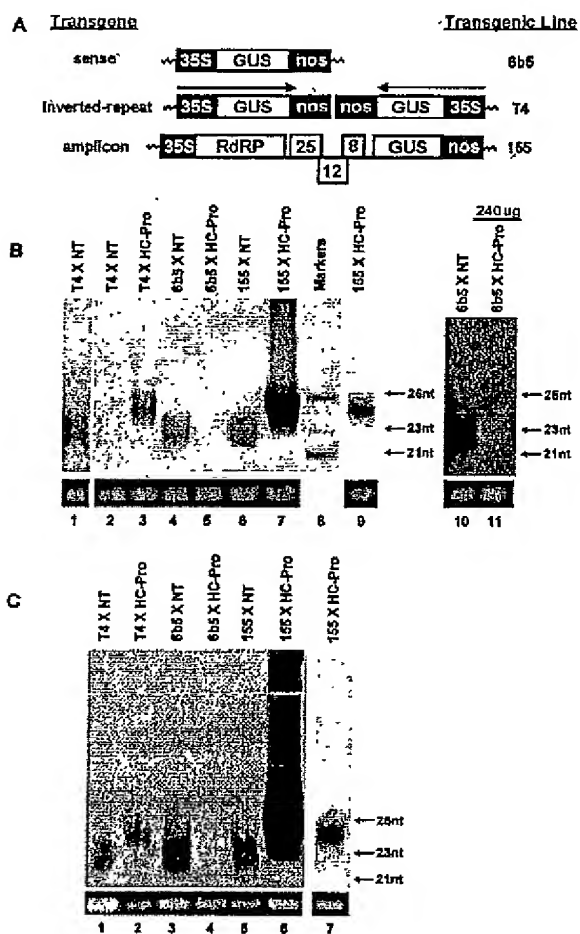


FIG. 1

Title: Compositions and Methods for the Modulation of Gene Expression in Plants
 Inventor(s): Vicki Bowman Vance
 Application No: To be assigned
 Atty Dkt No: 46759/250579

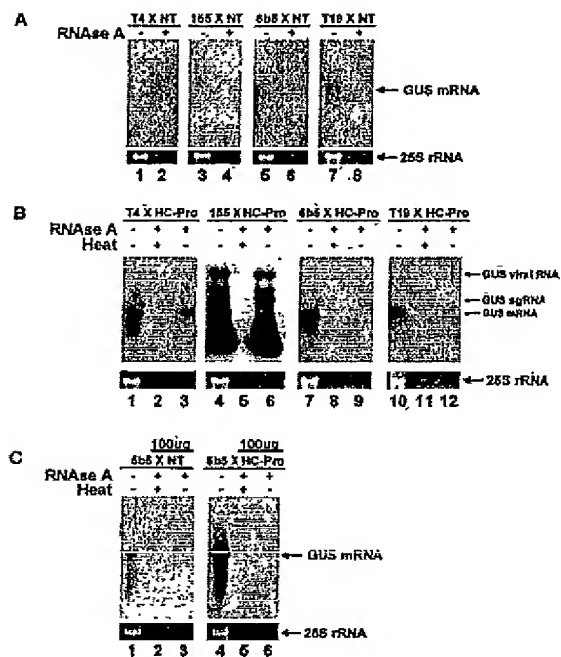


FIG. 2

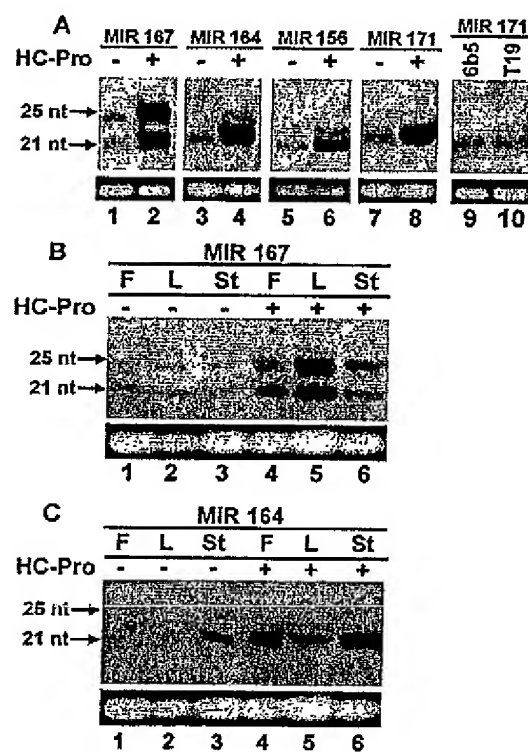


FIG. 3

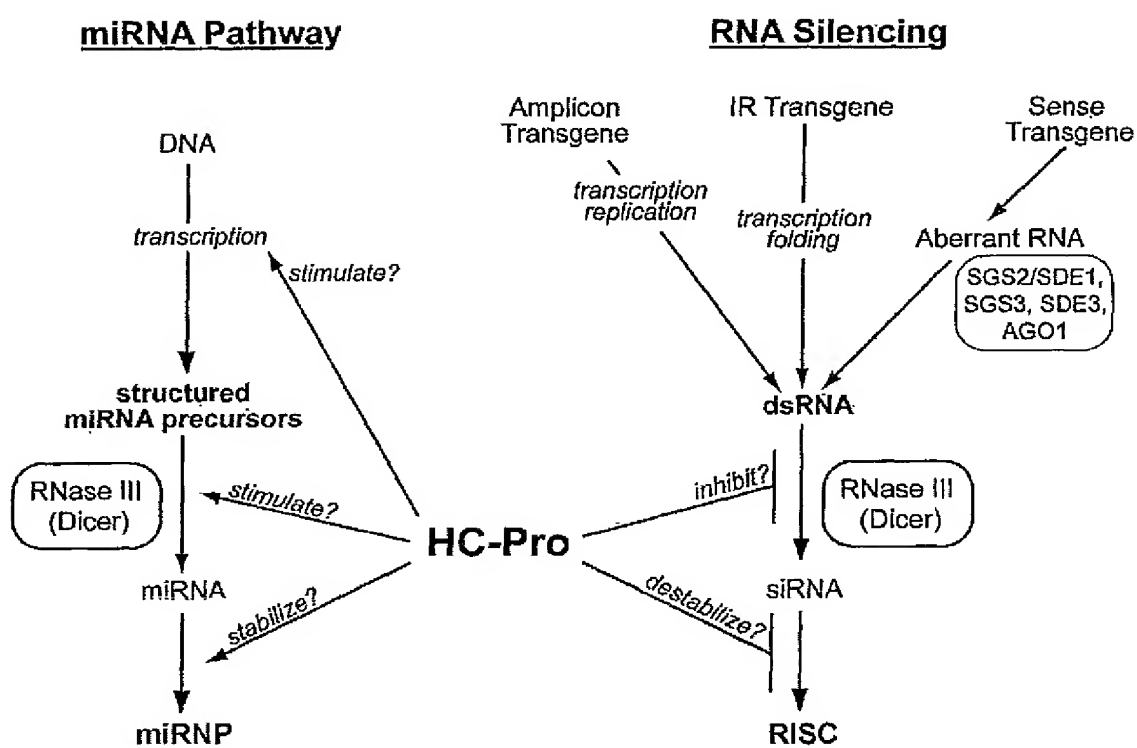


FIG. 4

Appendix B

Attorney Docket No. 9536-3

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Vance et al.

Application Serial No.: 10/623,930

Filed: July 21, 2003

For: *Compositions and Methods for the Modulation of Gene Expression In Plants*

Confirmation No.: 6465

Group Art Unit: 1638

Examiner: V. Kumar

**Declaration of Vicki Bowman Vance, Ph.D.
Pursuant to 37 C.F.R. § 1.132**

I, Vicki Bowman Vance, do hereby declare and say as follows:

1. I am a named inventor under United States Application No. 10/623,930 ("the '930 application") and of the subject matter claimed therein.

2. I have a Ph.D. in Plant Biology, from Washington University (St. Louis, MO). I am a Professor of Biological Sciences at the University of South Carolina, Columbia, SC. Presently, I am also serving as Program Director, National Science Foundation, Molecular and Cellular Biology Division. I am involved in research in the area of gene silencing in plants and have authored or co-authored more than 16 publications related to this area. A *curriculum vitae* is attached herewith at Appendix B.

3. The practice of this invention entails using natural plant genes that encode microRNAs (miRNAs) and modifying those genes using standard recombinant DNA technology, known in the art at the time of filing of the 093 application, so that "artificial" miRNAs are produced that target the RNAs of choice. MiRNAs are small RNAs (usually 21-24 nucleotides long) that incorporate into a large protein silencing complex and guide that complex to a particular target RNA and prevent the target RNA from producing protein, either by blocking translation or, more commonly in plants, by causing the target RNA to be cleaved. MiRNAs are not transcribed directly from their genes as 21-24 nucleotide small RNAs, but are made as longer precursor RNAs that contain a stem-loop structure in which the miRNA sequence is found within one arm or the other of the stem. The initial miRNA transcript is called a primary transcript (or the pri-miRNA) and usually contains sequences both upstream and downstream of the stem-loop structure. However,

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it is the stem-loop structure that is important for the proper processing events needed to produce a mature functional miRNA.

An intermediate step in miRNA production is the production of the stem-loop structure itself, which is called the precursor miRNA or pre-miRNA. MiRNAs and the stem-loop precursors from which they are derived were well known in the art at the time of filing of the present application. The primary literature describing plant miRNA precursors is cited in the application (Reinhart et al., *Genes Dev.* 16(13): 1616-26 (2002) and Llave et al., *Plant Cell.* 14(7): 1605-19 (2002); copies provided with applicants' response dated August 10, 2006). Reviews describing the structures of the precursors that are processed to produce the mature functional miRNAs have also been published (for example, Bartel and Bartel, *Plant Physiol.* 132(2): 709-17 (2003) and Kidner and Martienssen, *Trends Genet.* 19(1): 13-6 (2003); copies provided with applicants' response dated August 10, 2006). Thus, plant miRNAs and their precursors, as well as the technology used to isolate them, were well known in the art at the time of filing the '093 application.

4. The structures of 863 plant stem-loop miRNA precursors are currently available at a website described by Griffiths-Jones and colleagues (*Nucleic Acids Res.* 34(Database issue): D140-42006 (2006); copy enclosed at Appendix C). The first release of this database was in July of 2003 (Release 2.0). Only the stem-loop structure is reported, because the actual length of the primary transcript is not known in most cases. However, as stated above, it is the stem-loop that is the important part of the miRNA precursor. Thus, miRNAs and their precursors were well known in the art at the time of filing the present application, and this information was then and remains easily and publicly accessible.

5. The '093 invention describes a general strategy to make "designer miRNAs" (also referred to as "artificial miRNAs" or "synthetic miRNAs") that target cellular or viral RNAs making use of the natural miRNA genes cited above. Thus, the natural miRNA sequence within the natural miRNA gene is modified so that it is now the desired "artificial" miRNA. To ensure that the newly designed miRNA remains within the proper context of the natural miRNA precursor and is therefore properly processed, the sequences opposite (or complementary) to the artificial miRNA in the stem are also modified to maintain the secondary structure of the stem. The modifications needed to produce a stem-loop that is

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subsequently processed into an artificial miRNA utilize straightforward recombinant DNA technologies that were standard in the art at the time of filing of the '093 application.

Further, since the filing of the present application, the invention has been successfully practiced by several independent labs, each using a different miRNA gene as the backbone. The results of the successful use of the invention have been published in highly respected scientific journals and some of these are briefly described below.

a) The Vaucheret lab successfully demonstrated the making and using of the invention by modifying one of the genes that encodes miRNA 168 (miR168) in the model plant *Arabidopsis thaliana*. The miR168 gene was modified to change the natural miR168 sequence within the precursor to one targeting a messenger RNA different from the original miR168 target. The other side of the stem structure in the precursor was modified to maintain the secondary structure of the engineered miR168 precursor, as taught in the invention. The desired target was cleaved in transgenic plants thereby demonstrating the success of the invention. This work was published in 2004 (Vaucheret et al., *Genes Dev.* 18(10):1187-97 (2004); copy provided with applicants' response dated August 10, 2006).

b) The use of the present invention was recently demonstrated in a more global way in the Weigel lab (Schwab et al., *Plant Cell.* 18(5):1121-33 (2006); copy provided with applicants' response dated August 10, 2006). These investigators used two different miRNA genes (miR172a and miR319a) as backbones to produce designer miRNAs to target specific genes as taught by the invention. They refer to the designer miRNAs produced this way as artificial miRNAs (amiRNAs). The investigators reported that both miR172a and miR319a genes worked well for producing amiRNAs and that they were able to target a variety of endogenous plant genes in a very efficient and straightforward manner using the amiRNA technology.

c) The invention was also successfully used in a publication appearing in 2006 (Alvarez et al., *Plant Cell.* 18(5):1134-51 (2006); copy enclosed at Appendix D). These authors used the *Arabidopsis* miR164b gene as the backbone of their artificial miRNA. The artificial miRNA was targeted to a set of three endogenous *Arabidopsis* genes by designing an artificial miRNA that was complementary to a sequence common to the expressed messenger RNAs of all three *Arabidopsis* RNAs. Thus, the authors

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demonstrated that a single artificial miRNA could be used to target multiple RNAs. This publication concludes that "miRNAs provide a flexible, quantitative, and autonomous platform that can be employed for regulated expression of multiple related genes in diverse species."

d) A fourth publication demonstrates that the artificial miRNA invention can also be successfully employed to target viral RNAs that are required for the replication of certain plant viruses, thereby establishing resistance to the targeted virus (Niu et al., Nat Biotechnol. 24(11):1420-8 (2006); copy enclosed at Appendix E). In this published work, the authors used a miRNA gene encoding miR159 as the backbone for their artificial miRNA. As seen in the previous examples as well as in this case, the modifications to the miRNA gene, to modify the natural miRNA sequence to the desired artificial miRNA and, subsequently, to modify the opposite strand of the precursor to maintain proper secondary structure of the precursor, were techniques known in the art at the time of filing of the present application. These technologies are straightforward and successful and show that the invention works very well. These authors also point to the general applicability of the technology and the ease with which it can be used.

e) The Niu et al, 2006 reference also demonstrates the use of a "dimeric miRNA precursor" to produce two different artificial microRNAs from the same precursor. In this case, the authors first engineered two different artificial miRNA precursors using the miR159 stem-loop backbone and demonstrated the efficacy of each independently. Then they constructed a dimeric gene, which contained the two individual artificial miRNA constructs ligated together and under control of the same promoter. Thus, the two miR159 stem-loop structures, each containing a different artificial miRNA, were part of the same miRNA precursor. This dimeric precursor strategy was quite effective and allowed the investigators to produce two different artificial miRNAs in a coordinated fashion.

6. Finally, the Nui et al., 2006 publication also brings up a point that relates to the ease with which the invention can be practiced. The whole process can be pre-tested using a well-established standard transient assay called agro-infiltration. The agro-infiltration assay was developed early on for work on RNA silencing (Brigneti et al., EMBO

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J. 17(22): 6739-46 (1998); Johansen and Carrington, Plant Physiol. 126(3):930-8 (2001); copies enclosed at Appendix F) and was later very successfully adapted to the evaluation of endogenous microRNAs and their targets (Llave et al, Science. 297(5589):2053-6 (2002); copy enclosed at Appendix G). This standard assay can be used both to ensure that the artificial miRNA is properly produced from the modified gene and that the artificial miRNA will work to cleave the desired target. Thus, the success of the particular artificial miRNA can be quickly and easily assayed before proceeding to make stable transgenic plants. In the transient agro-infiltration assay, the binary plasmid carrying the modified miRNA gene is transformed into *Agrobacterium* and then the transformed bacteria are infiltrated into the leaves of *Nicotiana benthaminiana*. The accumulation of the desired artificial miRNA can then be assayed by standard technologies such as those described in these publications and well known in the art at the time the application was filed, including Northern gel blot analysis. Similarly, to determine if the artificial miRNA will cleave the desired target, the two constructs (one expressing the artificial miRNA and the other the putative target RNA) can be co-infiltrated into the same spot on the same leaf and the cleavage of the target at the miRNA target site assayed by standard methods, including Northern gel blot analysis and 5' Rapid Amplification of cDNA Ends (5'-RACE).

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Vicki Bowman Vance, Ph.D. 1/2/2007
Vicki Bowman Vance, Ph.D. Date

10. RELATED PROCEEDINGS APPENDIX

None